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June, 1938

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CORONAL CEMENTUM AND CORONAL RESORPTION[1]

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Among the tissue changes in human teeth still baffling to a large part of the dental profession and even to some men engaged in dental research are the resorptive and reparative processes occurring prior to eruption. It has long been known that resorption sometimes takes place on the crown or root of an embedded tooth; little information, however, is available concerning preeruptive changes on the crown surfaces of normally formed and erupted human teeth.

Noyes (1) refers to "coronal cementum" describing it as a deposition of small amounts of cementum on the occlusal fissures, which cementum is occasionally observed in ground sections through molars and bicuspids. Beyond this, dental literature contains very little concerning the frequency and possible etiology of such an occurrence.

In order to understand the problem we have to consider briefly the condition of a tooth after completion of amelogenesis and before eruption. The completed enamel is covered by several layers of stratified squamous epithelium, the reduced enamel epithelium, normally continuous over all of the enamel. In fully embedded teeth it may persist intact throughout the entire span of life. Occasionally, however, in embedded teeth, a dehiscence develops in the enamel epithelium, and then the connective tissue of the tooth follicle comes in contact with the enamel surface. Then one of two things is possible: The connective tissue may deposit cementum directly upon the intact enamel surface. Since this degeneration of enamel epithelium occurs most commonly near the cemento-enamel junction, most of these cementum deposits appear as so-called cementum spurs, extensions of the radicular cementum over the cervical portion of the crown. The other possibility is that the exposed enamel may be

[1] Presented at the Sixteenth General Meeting of the International Association for Dental Research, Minneapolis, Minnesota, March 13, 1938.

resorbed, followed by the deposition of bone or a bone-like substance to replace the enamel and dentin. The more or less extensive defects which are occasionally observed in completely embedded teeth belong in this category.

The defects in the epithelial attachments of erupted teeth and their sequelæ were first discussed by W. Meyer (2), who called attention to the relative frequency with which the reduced enamel epithelium (appearing as epithelial attachment to the enamel) becomes atrophic near the cemento-enamel junction. Orban (3) showed a case of resorption of the enamel near the cemento-enamel junction of an erupted human tooth. In the molars of hypophysectomized rats Schour (4) found extensive enamel resorption in the region of the epithelial attachment; sometimes these defects extended through the enamel into the dentin. Schour suggested as a possible explanation for this the delayed eruption of the molars in these animals, which may contribute to the atrophy of the epithelial attachment. Small cementum spurs at the cemento-enamel junction are a very common occurrence in all human teeth. Van Kirk (5) found the cementum overlapping the enamel in 67 per cent of 443 teeth. In embedded teeth these degenerative changes in the enamel epithelium are well known. Much less information is available concerning the possibility of such proc-

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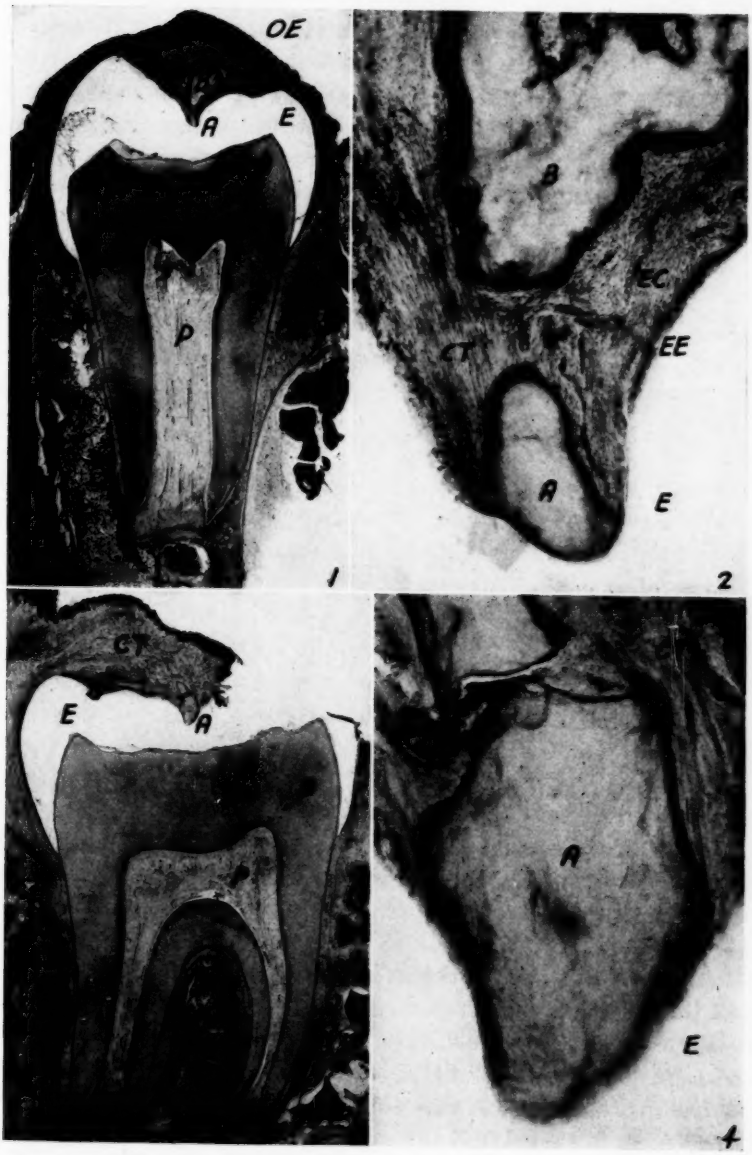
FIG. 1. Labiolingual section through an unerupted lower second permanent molar of a boy of 11 years. A round calcified mass lies in the connective tissue opposite the occlusal fissure. The bottom of the fissure is filled with a similar, smaller mass of coronal cementum. P, pulp; D, dentin; E, enamel space; AB, bone; A, coronal cementum in the bottom of the fissure; B, calcified body (cementicle) lying free in the connective tissue of the tooth follicle; OE, oral epithelium. Magnification × 4

FIG. 2. High magnification of area B in Fig. 1. E, enamel space; EE, remnants of enamel epithelium; CT, connective tissue of tooth follicle; EC, epithelial cells (remnants of outer enamel epithelium; A, coronal cementum in the bottom of the fissure; B, calcified body (cementicle) lying free in the connective tissue of the tooth follicle. Magnification × 64

FIG. 3. Mesiodistal section through a partially erupted lower second permanent molar of a boy of 11 years. Most of the mesial cusp is erupted; the rest of the crown is still united with the enamel epithelium. The occlusal fissure is filled by a large, irregular calcified mass which lies in direct contact with the enamel surface. P, pulp; D, dentin; E, enamel space; A, coronal cementum; CT, connective tissue of tooth follicle. Magnification × 4

FIG. 4. Higher magnification of area A in Fig. 3. E, enamel space; A, coronal cementum; CT, connective tissue. Magnification × 64



FIGS. 1–4

esses taking place in the still unerupted teeth of children. Yet the occasional observation of coronal cementum on the occlusal surface of normally erupted molars indicates that even during the relatively short intervening period (3 to 4 years) between completion of amelogenesis and eruption of the permanent teeth, a degeneration of the enamel epithelium and deposition of cementum are possible. In this report several such instances will be described.

In the routine examination of sections through tooth follicles of not yet erupted permanent molars, degeneration of the enamel epithelium and calcification of the epithelial cells are frequently observed. Sometimes these calcifications assume the shape of a sphere, lying free in the connective tissue opposite the occlusal surface and measuring from 1 to 2 mm. in diameter. Mummery (6) illustrates such calcified bodies in the tooth follicle. His statement, however, that they are always well separated from the inner enamel layers is not substantiated by the findings of the writer (*figs. 1 and 2*). In addition to the free calcified masses, there are cementum deposits on the enamel surface at the bottom of the fissure, in the field where Noyes described the coronal cementum in ground sections. In some areas more than one-half of the depth of the fissure is filled. The cementum lies directly in contact with the enamel; occasionally there is an indication of a cuticle between enamel and cementum. *Figs. 3 and 4* show a large irregular mass of calcified tissue occupying the fissure of a lower second molar and protruding nearly 1 mm. above the level of the surrounding enamel. Obviously the presence of such a rough, prominent irregularity in the enamel must considerably increase the caries liability of the molar after its eruption. Through the courtesy of Dr. I. Schour, of Chicago, the writer is in a position to illustrate an unusually clear case of coronal cementum in the occlusal fissure of an upper molar (*fig. 5*). There appears to be coronal cementum in many ground sections, but the small quantity of material in the fissure and the presence of particles resulting from the grinding process usually make it difficult to distinguish between true coronal cementum and mere débris. In *fig. 5* there can be no doubt as to the nature of the material contained in the fissures.

The etiology of the spherical calcification lying free in the connective tissue of the tooth follicle is closely related to the formation of

cementicles in the periodontal membrane. In both instances we are dealing with a degeneration of epithelial tissue and subsequent encapsulation and infiltration of the degenerated cells by calcific deposits (Gottlieb (7)). This is very clearly visible in *figs. 6 and 7*, which show the process of calcification in the tooth follicle of a completely embedded upper third molar of a man of 32 years. This molar shows all the various forms of calcifications in the tooth follicle.

FIG. 5. Ground section through an intact upper human molar. Higher magnification of the occlusal fissure. E, enamel; A, coronal cementum with numerous cementum lacunae; B, débris. (Courtesy of Dr. I. Schour.)

The bottom of the enamel fissure is lined by a thin layer of cementum. Opposite the fissure an irregular calcified body, measuring about 3 by 2 by 2 mm., lies in the connective tissue. It is surrounded by numerous small, round calcified bodies which apparently are the result of calcification around degenerated epithelial cells; they very closely resemble cementicles in their early stages of formation. The bottom of the fissure is covered by a continuous thin layer of cementum. In addition there are several irregular areas of calcification scattered

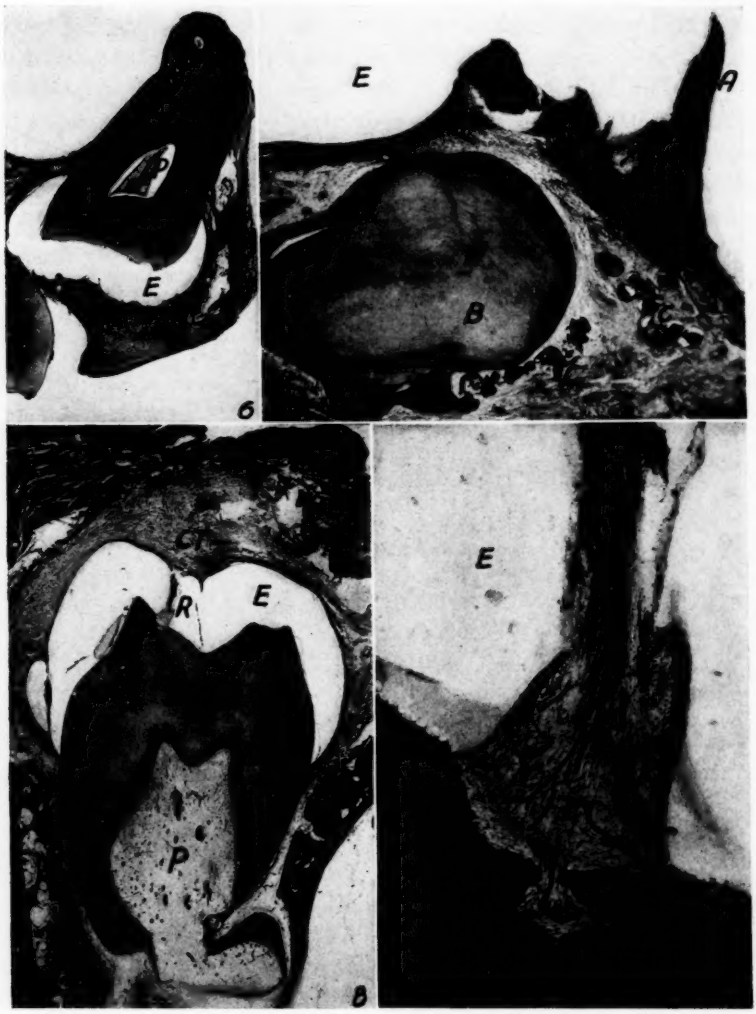


FIG. 6. Mesiodistal section through the crown of a completely embedded upper third molar (Logan, J. A. D. A., **23:** 854, 1937). A large, irregular calcified mass is located in the connective tissue opposite the center of the occlusal surface. The bottom of the fissure itself is covered with coronal cementum. Several smaller calcified bodies are scattered through the connective tissue and the enamel epithelium. P, pulp; D, dentin; E, enamel space; B, large, round calcified body located in tooth follicle. Magnification × 2.5

through the enamel epithelium. They are located intraepithelially, and some of them are surrounded by a secondary cuticle. These calcifications within the enamel epithelium are, of course, essentially different from true cementum; cementum can be formed only by connective tissue and not by epithelium. But they are an additional support to Gottlieb's observation that epithelium of dental origin shows a marked tendency to become calcified if there is degeneration.

Another interesting finding in unerupted teeth is the occurrence of resorption of the crown. *Figs. 8 and 9* show the unerupted lower second permanent molar of a child, aged 8½ years. Near the tip of a lingual cusp a deep, narrow defect runs through the entire thickness of the enamel and terminates in the superficial layers of the dentin. It contains loose connective tissue and numerous capillaries. The bordering dentin shows the typical contours of Howship's lacunae. On the corresponding tooth of the opposite side of the mandible, a similar resorption is present which has been extensively repaired by the deposition of an irregular calcified material in the defect. In some areas channels containing connective tissue and blood vessels are still present within the calcified substance; they are connected with the tooth follicle by a narrow, tortuous duct through the enamel. With the exception of a publication by Pelzmann (8) who showed a resorptive defect and reparative hard tissue formation in the crown of an unerupted tooth, the writer has been unable to find any report in the literature showing the development of preeruptive resorptions in sections through unerupted teeth of children. There are, however, several cases on record in which repaired resorptions have been observed in the histologic examination of erupted teeth, with or without

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FIG. 7. Higher magnification of the occlusal surface in Fig. 6. E, enamel space; A, coronal cementum covering the enamel of the fissure; B, large, round calcified body (cementicle); C, small cementicles, surrounding cell débris; EE, enamel epithelium, containing small irregular calcifications. Magnification × 30

FIG. 8. Labiolingual section through the tooth germ of a lower second permanent molar of a boy of 8½ years. P, pulp; D, dentin; E, enamel space; R, resorption channel extending through the enamel into the dentin; CT, connective tissue of tooth follicle. Magnification × 4

FIG. 9. Higher magnification of area R in Fig. 8. E, enamel space; D, dentin; CT, loose connective tissue with numerous capillaries, extending through the enamel; R, resorption of the dentin. Magnification × 70

gross clinical abnormalities. *Figs. 8 and 9* show how such resorptions originate.

To the question as to why the enamel epithelium sometimes degenerates, and why then one time apposition and another time resorptions occurs, there is as yet no satisfactory answer.

SUMMARY AND CONCLUSIONS

1.— So long as the enamel of an unerupted tooth is covered by the intact enamel epithelium, there is no possibility of change on the enamel surface. Only in areas where the enamel epithelium degenerates and disappears can cementum be deposited or resorption take place. This is true of the unerupted teeth of children as well as of embedded teeth in adults. 2.—Degeneration of the enamel epithelium next to the cemento-enamel junction is a common occurrence, which can take place even after eruption; this explains the frequency of cementum spurs extending from the cemento-enamel junction a short way over the enamel. 3.—Degeneration of the enamel epithelium on the occlusal surface may occur in the molars of children during the physiologic interval between completion of enamel and eruption. It is usually followed by calcification of the epithelial cells and formation of various types of hard tissue. 4.—Deposition of cementum upon the enamel surface produces true coronal cementum. It is most commonly found in the occlusal fissures of molars, probably because in the fissures the physiologic involution and atrophy of the enamel epithelium begin sooner than in any other part of the crown. 5.—In addition to the formation of coronal cementum, small and large, irregular cementicles form in the connective tissue of the tooth follicle. They seem to originate in the same way as the cementicles of the periodontal membrane, by calcification of degenerating epithelial cells. 6.—Sometimes small intraepithelial calcified masses are found within the reduced enamel epithelium. Nothing is known about the origin of this form of calcification; it also seems to be the outcome of degenerative processes involving small groups of epithelial cells. 7.—On rare occasions preeruptive resorptive processes may take place in areas of the enamel surface which have lost their epithelial covering. Such resorptions may extend through the entire thickness of the enamel into the dentin. Subsequent depo-

sition of hard tissue (cementum) may be expected in the resorbed area. Clinically, teeth with such preeruptive resorptions can be expected to show deep, irregular defects in the enamel; decay can be expected to start quickly in such defects unless they are filled shortly after eruption of the tooth.

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EFFECTS OF DAILY USE OF FLAVORED SODIUM PERBORATE AS A DENTIFRICE FOR A SIX MONTHS PERIOD[1]

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Sodium perborate is a medicament commonly used in the treatment of Vincent's infection. To prevent recurrence of the condition many individuals continue to use sodium perborate, either as a mouth wash or as a dentrifrice. Recently the belief has been growing that sodium perborate should be used only under the supervision of a dentist and never for a prolonged period. The harmful effects that have been ascribed to it are chemical burns, an edematous condition of the oral mucosa and "hairy" tongue. If the prolonged use of sodium perborate does result in any or all of these conditions, the dental profession should take steps to discourage its promiscuous use. It seemed worthwhile therefore to make carefully controlled observations of the effect of sodium perborate on a large group of persons.

EXPERIMENTAL TECHNIQUE

One hundred students agreed to use flavored sodium perborate as a tooth powder at least twice a day for a period of 6 months. The composition of the preparation used is sieved sodium perborate 99.5 per cent, calcium phosphate 0.5 per cent, colored with magenta, and flavored with menthol, cloves, eucalyptol and peppermint. The students were given no specific instructions as to the manner of brushing. By omitting instructions it was considered that the experiment would duplicate more closely the conditions under which the public buys and uses its dentifrices. The technique recommended

[1] Read at a meeting of the New York Section, Jan. 29, 1938 and in abstract at the General Meeting of the International Association for Dental Research, Minneapolis, March 13, 1938.

was that one half to one gram of powder should be poured into the palm of the hand, then the moistened tooth brush dipped into the powder, the teeth cleaned and the gingivae massaged. The duration of the brushing was 1 minute, as timed by a watch. A calendar was kept in the bathroom of all students so that a record could be made each time sodium perborate was used. Notarized agreements were filed at the beginning of the test period by all members of the group, stating that they would follow the above prescribed instructions. At the end of the test period, an affidavit was submitted by each student affirming that sodium perborate had been used faithfully with the exception of indicated periods.

The 100 students were divided into 3 groups, and the following procedure was carried out: A). [20 students] color photographs, blood smears and gingival biopsies were made; B). [37 students] color photographs and blood smears were made; C). [43 students] color photographs were taken.

Photographs were made in natural colors with Dufaycolor films of the gingivae and anterior teeth of all students at the beginning and at the end of the test period. A number of pictures of tongues were also included. Any pathological condition such as chemical burns, "hairy" tongues or edematous condition of the lips which might appear during the test period could be recorded by a second photograph and compared with the original condition. It was thought that this method of procedure had the advantage of presenting concrete and permanent evidence which could be demonstrated to all who would be interested in this subject. The purpose of the gingiva biopsies was to determine the condition of the tissue before beginning use of sodium perborate. If chemical burns should appear in Group A., a second biopsy would be taken to show the lesion and structural changes which might have been caused by the use of sodium perborate. Blood smears were made in order to obtain the eosinophile count, which might indicate an allergic state.

The experimental work was started in April, 1937 and was ended in November, 1937. The group selected for this study was composed of 100 students (98 male and 2 female). Two male students who had been included in the project discontinued their study of dentistry and their whereabouts could not be ascertained. As they had used sodium perborate for a period of 2 months without any reaction being

observed while they were under supervision, the records have been retained, the original group of 100 thus being included.

A questionnaire filled out by the members of the project gave information concerning the following points: 1) previous use of sodium perborate; 2) as a mouth wash or dentifrice; 3) length of time; 4) sensitivity to specific proteins such as strawberries, tomatoes, fish, chicken, veal, and to certain drugs such as laxatives, aspirin, etc.; 5) whether afflicted by hay fever, asthma, or hives. A final statement executed before a notary public was filled out by the students giving the following information: 1) the number of times sodium perborate was used per day; 2) the number of times the use of sodium perborate was omitted in the 6 months period; 3) whether abnormal effects or improvement in oral conditions had been noted.

RESULTS

The students watched the condition of their mouths very closely because their studies made them strongly "tooth conscious." This self-supervision proved satisfactory for the original purpose of the project, i.e., to ascertain whether harmful effects resulted from the continued use of sodium perborate. In case any students had discomfort or noted any bleeding from the gums they were examined.[1] Eighty-one of the 100 members of the experimental group did not report for examination; it was therefore considered that they had no discomfort nor were conscious of bleeding of the gums. Nineteen reported with various types of oral conditions. A summary of these symptoms shows that 3 students had signs of Vincent's infection; 4 showed gingival lesions, suggestive of tooth brush trauma. (Gingival bleeding, hyperemia and tenderness always accompanied the above conditions.) In addition to these lesions, the following transient conditions were noted. One student showed gingival bleeding; 2, gingival tenderness; 3, gingival inflammation due to infected retromolar flaps, around partially erupted third molars; 2, hyperemic gingivae; 2, gingival recession; 1, sensitive "necks" of teeth; and 1, fetid breath, the latter condition lasting for 3 days. Similar conditions, including Vincent's infection, were noted in a group of 53 students, who were not using sodium perborate.

The information obtained from the questionnaire indicated that

[1] By Dr. Daniel Ziskin or one of the authors (L. R. C.).

17 per cent of the group had previously used sodium perborate; 8 per cent of these had used it as a mouth wash; 6 per cent as a dentifrice and 3 per cent in both forms. The length of time sodium perborate had been used ranged from a few days to 4 years. Sensitivity to specific foods and drugs was present in 7 per cent; 9 per cent suffered from hay fever; 3 per cent from asthma and 6 per cent from hives.

The final statement executed before a notary public showed that sodium perborate had been used twice a day, 2 students even brushing their tongues. One member brushed his teeth 3 times a day with the reagent. Fifty-one per cent of the students used sodium perborate continuously. Forty-nine per cent testified to the following lapses:

No. of brushings missed.....	1–5	6–10	11–15	16–20	21–25	26–30	36–40	56–60	61–65	91–95	Indefinite	
No. of students	5	16	11	4	4	3	1	1	2	1	1	49

Analysis of this table shows that 36 of 49 students missed less than 20 of 360 prescribed brushings.

Abnormal oral conditions, lasting for short periods were reported by 19 per cent of the members of the project. Seventeen per cent of the students noted an improvement in their mouths, while no effects were observed by 64 per cent of the group.

DISCUSSION

The purpose of the investigation was to determine whether chemical burns, "hairy" tongue, or edematous conditions of the oral mucosa resulted from the twice daily use of sodium perborate. Before discussing the results of the experiment it is necessary to define these pathological conditions.

A burn is usually associated with the application of heat, but contact with corrosive liquids such as sulphuric acid, nitric acid, and caustic potash, produces lesions which show practically the same pathological and clinical features as those caused by heat. The term "burn" has many definitions.[3] Among these we find the phrases: "to become inflamed"; "to have the sensation of heat"; "to oxygenize"; or to "to cauterize." Consequently any injury of

[3] Gould's Medical Dictionary (3rd edition).

the oral mucous membrane not due to physical trauma may be called a burn.

Gingival trauma results in lesions which at first appear to be similar to chemical burns. Four students showed this condition, but further observations indicated that these were not chemical burns. As the subject of "burns" is frequently discussed in the dental literature, it seems advisable at this time to analyze the factors which might be suspected of causing or simulating chemical "burns" of the gingivae. They are: 1) allergy; 2) tooth brush trauma; 3) high concentration of solution; 4) prolonged contact with the mucosa.

1) Allergy: In the last few years a great deal has been heard concerning the term *allergy*. It is used to explain a series of skin and mucous membrane changes and lesions that can in no other way be accounted for. Frequently, and unfortunately too frequently, there is no scientific basis for the use of this term. Drug rashes, excoriations and eruptions are often called allergic. The mechanism involved is not known and it is not often possible to show that the skin is hypersensitive to the drug involved. In the case of some drugs, the hypersensitivity is in the epidermis while in the majority of instances the dermis is the site of change. Most drug allergies are due to the ingestion of the drug and not to contact with it. The lesions noted vary from an erythematous blush to actual bullae fungations and ulcerations.

Sodium perborate is used as a topical remedy, so that most of its ill effect would be in the form of a contact dermatitis or *dermatitis venenata*. There are 2 factors to consider when viewing this effect of sodium perborate. Are the lesions the result of a burn or are they due to trauma? If due to the former, they are not allergic because the injury is chemical, similar to an injury of the skin with a strong alkali or caustic. The traumatic effect will be discussed below.

The subject of allergy can well be summed up in the words of Becker (1) as follows: "There is not enough evidence at the present time to consider drug eruptions as allergic diseases."

2) Tooth brush trauma of the gingivae. Tooth brush trauma of the gingivae is another cause of gingival lesions which have the appearance of chemical burns. The sensitivity of gingival tissue to traumatic injury varies. In some persons the resistance of this tissue is

so high that it is not injured even when the brushing of the teeth is energetic enough to break the handle of the tooth brush. This extraordinary resistance, however, is rare. More often the gingivae are unduly sensitive, so that fragments of a bread crust or an incorrect method of brushing the teeth causes injury; even the use of a moistened tooth brush without a dentifrice can cause gingival trauma if this tissue is sensitive. Our observations of the experimental group of the sodium perborate project showed 4 students having lesions with the appearance of chemical burns. Three of these were due palpably to tooth brush trauma as they occurred in areas of the gingivae which were prominent because of the mal-position of the teeth. Further experimentation indicated that even the fourth case did not appear to be a burn. All 4 students who showed this condition were told to discontinue the use of sodium perborate temporarily. After their return from summer vacation they resumed the use of sodium perborate for a period of 4 weeks. The fact that no burns or lesions of any kind recurred strengthened the belief that the cause of the original lesion was physical trauma. One student who had developed a lesion on the lingual gingiva near the lower premolars had learned the proper method of brushing in class. He, himself, ascribed the formation of the lesion occurring at the beginning of the project to faulty brushing.

3) Concentration of Sodium Perborate. The concentration of this reagent is the third factor which may be responsible for chemical burns appearing on the gingivae. Therefore a comparison was made of the strength of the solutions of sodium perborate when employed

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DESCRIPTION OF FIGURES 1–4

FIG. 1. Gingival and buccal mucosa before starting experiment.

FIG. 2. Same individual as *fig. 1* taken after completion of experiment. There is no appreciable difference in appearance of tissue.

FIG. 3. A picture of tongue taken before starting experiment. This tongue shows irregular coated patches giving appearance of geographical tongue. Note how far student can protrude tongue. This is usually significant of apprehension and indicates constant observation of organ by patient.

FIG. 4. Picture of same tongue taken after completion of experiment. There is no change in its appearance. A slight degree of coating is perfectly normal. This is due to enmeshing of desquamated epithelium and coating tends to increase if tongue is not used sufficiently in eating and talking.

FIG. 2

FIG. 1

FIG. 4

FIG. 3

as a mouth wash and as a tooth powder; the latter was found to be slightly higher. In order to estimate the concentration it was necessary to ascertain the quantity of powder used, the amount of fluid present in the mouth, and that in a moistened tooth brush. The following figures give approximate ranges: the average amount of sodium perborate taken up on a commonly used tooth brush the bristle ends of which occupy 3 square centimeters varies from 100 to 700 milligrams. This amount is dissolved by the saliva present in the mouth, which is shown by tests on 5 persons to vary from 0.5 to 1.8 cc. If the mouth is rinsed before brushing the teeth, a common habit with many persons, the residual oral fluid remains about the same. The water in the moistened tooth brush must also be added. This averages 700 milligrams making a total amount of fluid 1.2–2.5 ccm. which suspends the 100–700 milligrams of sodium perborate.

The act of brushing the teeth stimulates the flow of saliva; this is more profuse in some persons than in others. Dr. Maxwell Karshan states that an average amount of 1 ccm. of saliva is secreted per minute by chewing paraffin. There is probably no doubt that the act of chewing stimulates the salivary glands more than does the brushing of the teeth. If 1 cc. of fluid is added to the solution in the mouth in this manner during 1 minute of brushing, the total amount ranges between 2.2–3.5 ccm.

Sodium perborate is sparingly soluble in water (1 in 40 parts of water at 25° centigrade). Consequently a concentrated solution of the reagent would occur in the mouth if only 100 miligrams of powder were taken on the brush and dissolved in 4 ccm. of fluid. As the maximum total amount of saliva and water is calculated to be only about 3.5 ccm., it is clear that the oral fluid usually remains saturated with sodium perborate during the entire brushing period.

A slightly weaker solution of the reagent is used usually as a mouth wash for the treatment of Vincent's infection. Common instructions to patients are to use 1 teaspoonful to three-quarters of a glass of water. The concentration of this solution varies considerably, depending on the amount of powder taken on the teaspoon and the size of the glass used. A level teaspoonful contains 90 grains of powder and if an 8 oz. tumbler is used and filled three quarters full, it will contain 6 oz. of water. The concentration of sodium perborate would then be 2.08 per cent.

A comparison of the strength of the solution used as a mouth wash and as a dentifrice shows that both nearly approach the saturation point. The fact that the sodium perborate is slightly stronger when used as a dentifrice than as a mouth wash indicates that the conditions of the experiment constituted a more drastic action of the reagent.

4) Prolonged Duration of Contact. When sodium perborate is used as a tooth powder, it is estimated that the time of contact is not longer than a few minutes. The actual time that most people brush their teeth varies probably from 3 minutes to 30 seconds or less. Usually the mouth is rinsed after the completion of the brushing, and in this way all but a trace of sodium perborate is removed. On the other hand, the contact of the sodium perborate solution is probably more prolonged and its distribution more general, when it is used as a mouth wash. Patients who have had the disagreeable symptoms common to Vincent's infection naturally wish to hasten the cure; consequently it is likely that some may retain the solution in the mouth much longer and use it more often. Possibly this is the reason that chemical burns are reported following the use of sodium perborate.

The routine use of a chemical may produce no effect whatsoever on the tissue; however one author (6) filled a small cloth bag with sodium perborate and placed it inside the cheek for one half hour. A burn is reported to have appeared by this means. It is clear that such a manner of application bears no relation to the routine use of sodium perborate, hence it does not seem logical to say that the tissue was hypersensitive to this material simply because it had become irritated and inflamed when in long contact with a concentrated form of the chemical. We should like to reiterate that a burn is dependent chiefly upon the physical state of the tissue to which it is applied, and the concentration of the solution.

Still another possible factor exists which may be responsible for the effect of sodium perborate on the oral mucous membrane, i.e. the age of the user. Our group was composed entirely of students whose ages ranged between 19 and 30 years; the average being 23.92 years. However, it seems hardly likely that older persons are more prone to chemical burns than young ones.

The "Hairy" Tongue: Of late there has been considerable dis-

cussion concerning a clinical curiosity known as "hairy" tongue. Several observers have noticed the occurrence of what they call a "hairy" tongue after the use of sodium perborate and this has popularized this condition. The term "hairy" tongue has been more or less loosely used to define all types of pigmentation, furring, and coating of this organ. In order to understand the true picture we should have a thorough knowledge of the underlying pathology.

The most complete work on this subject was reported by Heidingsfeld (2). He states that hairy tongue can be conveniently divided into 2 general classes. One group consists of the true idiopathic or genuine cases characterized by well-defined, stable black-brown or yellow-brown, thick, soft, fur-like patches, covered with densely intertwined hair-like filaments measuring from $\frac{1}{4}$ to $\frac{1}{2}$ inch in length; and the second group is composed of false or pseudo-spurious cases, characterized by thick yellow-brown or greenish discoloration of unstable evanescent character covered with a soft mushy detritus, occasionally containing short filaments measuring $\frac{1}{8}$ to $\frac{1}{4}$ inches in length. He believes that the true cases owe their origin to some anomaly of development in the sense that the germinal products, from which they are developed, are present from birth but do not undergo growth and developmental changes until early adolescence or at some later period in life. This is somewhat confirmed in the report of a case (3) of an infant entirely breast-fed, 15 months old, affected with hairy tongue. The tongue had not been subjected to any form of irritation. Heidingsfeld (2) compares the true hairy tongue to conditions such as cutaneous horns,—ichthyiosis hystrix, —etc. There has been very little investigation on the complete histology of true hairy tongue. This has been due to the fact that very few patients are willing to submit to the excision of enough material for study. Most of the histological examinations have been confined to microscopical study of removed filaments. However, there are 3 instances in which a piece of tongue has been excised and through these we are able to make a study of the histological changes. One of these specimens was removed by Heidingsfeld from a case of epithelioma of the tongue, apparently not related to the "hairy" condition; another was removed at autopsy and reported by Spencer and Cade (4). These sections show that the hairy effect is due to a hyper-

keratinization and elongation of the epidermis of the filiform papillae. In these cases the picture is not unlike that of an exaggerated epithelial papilloma. In furred or pseudo-hairy tongues the papillary elongations are not as exaggerated as one sees in the true instances, and the tongue is apparently coated with a material in which is included desquamated epithelium, bacteria, yeasts, and detritus in general. The elongation of the papillae is an inflammatory hypertrophy.

Many causes have been suggested for pseudo hairy tongue. Among them may be mentioned tobacco, antiseptics, astringents, wine, gastric upsets, etc. Syphilis was thought to be of some etiological importance since many of the earlier cases reported were in luetics, but syphilis evidently plays a minor role. The condition in such patients probably was brought about by intraoral medication for the relief of syphilitic lesions. There seems to be little doubt that the pseudo hairy tongue cases are the ones that may occur following the use of sodium perborate.

Since so large a number of etiological factors has been incriminated it might be well to state that the continuous use of any medicated mouth wash may cause an inflammatory hypertrophy of the tongue papillae, but it is unscientific to lay emphasis on one type of drug alone, especially since there has been no apparent effort to rule out other contributory causes.

SUMMARY

The purpose of the investigation was to determine whether the prolonged use of flavored sodium perborate caused chemical burns, hairy tongue or edematous conditions of the oral mucosa. The experiment was conducted on 100 students, age range 19 to 30 years, who agreed to use sodium perborate faithfully, twice daily as a dentifrice for 6 months. The teeth were brushed and the gum massaged for a period of 1 minute as timed by a watch. All mouths were photographed in colors in order to have permanent evidence of any subsequent pathological changes; gingival biopsies of 20 and blood smears of 37 students were made. This elaborate set up was arranged in the expectation that pathological conditions would be observed as a result of the use of sodium perborate. Observations showed 4

students with lesions, suggestive of tooth brush trauma, which did not reappear when the use of sodium perborate was resumed. No pathological conditions were noted which could be defined as chemical burns of the oral mucosa, hairy tongue or edema of the mucosa. This work is being continued.

CONCLUSION

Flavored sodium perborate was not harmful to the oral mucosa of the experimental group when used as a dentifrice twice a day for an extended period.

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TECHNIC FOR INTRA-ORAL RADIOGRAPHY OF LIVING DOGS

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Much experimental work is being done on dogs to observe and demonstrate changes in alveolar bone and dentition. Radiography would be a valuable aid during the course of experimentation if a suitable technic were available. The anatomical variations and difficulties encountered with living animals have probably been largely responsible for the failure to use radiography to its best advantage. The technic to be described embodies a definite system of angulation, film packet placement and retention, and exposure. The systematic procedure employed makes possible duplication of radiographs for comparative studies. Fourteen intra-oral views constitute a complete dental radiographic examination for dogs, 7 each for the maxillary and mandibular regions (*fig.* 7).

The angulations given are averages; modifications may be necessary. The average angulation will usually suffice when the teeth and jaws are fully developed and of average size. When the teeth are large, or the root formation and alveolar process is incomplete, a greater angulation will be necessary to avoid excessive distortion of the images. While any standard dental x-ray units may be used satisfactorily, a longer target film distance than that available on these machines is more desirable. The factors with which this technic was worked out are: 65 K. V. P., 20 Ma., 12 inches target film distance. The exposures tabulated are based upon the above factors and using standard 5 minute developer at 65 degrees Fahrenheit. Any changes in the above factors must be compensated for accordingly.

In order to assure complete immobility and to prevent interference from the subject, a suitable anaesthetic must be used. For this purpose sodium pentobarbital (Nembutal) administered intravenously has been found most desirable. Dosage is computed by weight and

the drug should be administered cautiously. The examination may be started 3 to 5 minutes after the injection. The services of 2 persons are required to adequately make the radiographic examination. One person should adapt and retain the film packets, while the other should center the tube and make the exposure. If extensive examinations are to be made, it is suggested that the persons alternate in the procedure so that excessive radiation to any one person will be minimized. For posing the subject a standard dog surgery table is recommended. Calibrated head positioners or similar devices for maintaining a fixed head position may be employed, but if extreme care is taken to place the head in the exact positions given, satisfactory results may be obtained and duplications may be accurately made for all practical purposes without mechanical aids.

TECHNIQUE

MAXILLARY INCISOR REGION. *Position of head*: The dog is placed on his back and head positioned so the long axes of incisor teeth are horizontal. *Angulation:* Vertico-horizontal angle: (angle with long axes of teeth). 35 degrees superior. Mesio-distal angle: Directly between 1st incisor teeth. *Position of film packet:* With the long dimension of the film packet corresponding to the long axes of the incisors, sharply curve the upper corners and closely adapt the packet to include all the incisor teeth. Have the lower edge of the film packet uniformly 4 to 6 mm. beyond the incisal edges of the teeth. *Retention of film packet:* The film packet is retained with the index finger in the center of the upper third, resting the thumb on the side of the maxilla. *Position of cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* 4½ seconds (regular films).

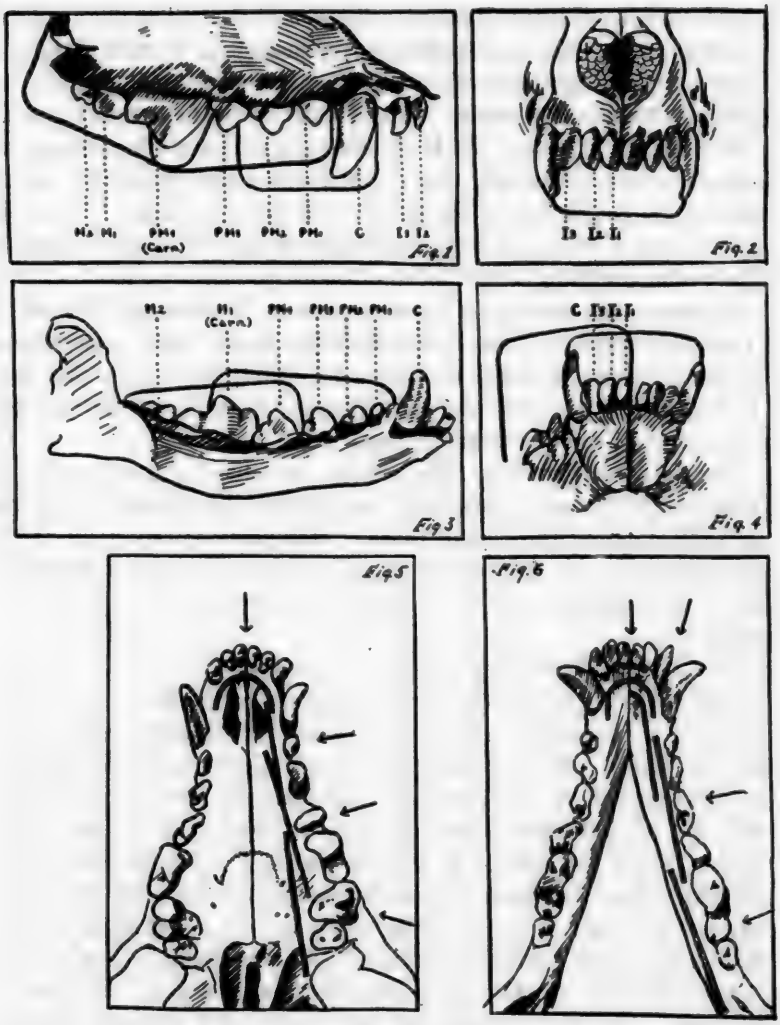
General considerations: If the teeth and alveolar process are fully formed and average size, the average angle of 35 degrees will suffice. However, if the teeth are exceptionally long, or the jaws and the root formation is incomplete a greater angulation must be used. If the arch is very narrow and constricted, it may be necessary to secure additional views of this region to obtain accurate images of the third incisors. *Figs. 2 and 5* illustrate the technical features described.

MAXILLARY CANINE REGION. *Position of head:* The dog is placed on his side and the head positioned so the side to be radiographed is horizontal. *Angulation:* Vertico-horizontal angle: 45 degrees superior. Mesio-distal angle: Perpendicular to the film packet (*fig. 5*). *Position of film packet:* With the long dimension of the film packet corresponding to the long axes of the teeth, closely adapt the packet to include the crown of the canine and extend distally. Have the lower edge of the packet approximately 4 mm. beyond the cusp of the canine and 8 to 10 mm. beyond the cusps of the premolars. *Fig. 1* illustrates the position of the film packet. *Retention of film packet:* The film packet is retained with the index finger in the upper mesial corner, resting the thumb over the nose. *Position of cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* 3½ seconds (regular films).

General Considerations: The length and distal curvature of the root and the low flat palatal bone are the chief difficulties encountered in radiographing the canine tooth. Modification, especially of the mesio-distal angle, will frequently be necessary in order to obtain an accurate image of the canine. Refer to *fig. 7* for the resultant radiographs.

MAXILLARY PREMOLAR REGION. *Position of head:* The dog is placed on his side and the head positioned so the side to be radiographed is horizontal. *Angulation:* Vertico-horizontal angle: 45 degrees superior. Mesio-distal angle: Perpendicular to the film packet, as illustrated in *fig. 5*. *Position of film packet:* With the short dimension of film packet corresponding to long axes of the teeth, closely adapt the packet to include the first premolar and extend distally. Have the lower edge of the film packet uniformly 5 to 6 mm. beyond the cusps of the premolar teeth (*fig. 1*). *Retention of film packet:* The film packet is retained with the index finger on the upper third, resting the thumb over the canine region. *Position of the cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* 4 seconds (regular films).

General considerations: One view is usually sufficient for examining the premolar region. When the teeth are large and widely spaced it may be necessary to use a longer film or make 2 exposures in order



FIGS. 1 to 6. Diagrams to show various positions of film packets

to include all the pre-molar teeth. This region is probably the least difficult to examine in that the teeth have comparatively short roots, as illustrated in *fig.* 7.

MAXILLARY MOLAR REGION. *Position of head:* The dog is placed on his side and the head positioned so the side to be radiographed is horizontal. *Angulation:* Vertico-horizontal angle: 50 degrees superior. Mesio-distal angle: 15 to 20 degrees distally. Refer to *fig. 5*. *Position of film packet:* With the short dimension of film packet corresponding to long axes of the teeth, closely adapt the packet to include all the third molar region and extend mesially. Have the lower edge of the film packet uniformly 2 to 3 mm. beyond the occlusal surfaces of the molar teeth (*fig. 1*). *Retention of film packet:* The film packet is retained with the index finger in the upper third, resting the thumb over the premolar region. *Position of cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* 5½ seconds (regular films).

General considerations: The molar region is probably the most difficult to radiograph due to the anatomical limitations. In order to avoid superimposition of the malar process and bone a distal shift of approximately 15 to 20 degrees with the tube must be made. This angulation will usually produce an undistorted image of the second molar. Due to the length and divergence of the first molar roots, it may be necessary to secure additional views of this tooth.

MANDIBULAR INCISOR REGION. *Position of head:* The dog is placed on his back and the head positioned so the long axes of the incisor teeth are horizontal. *Angulation:* Vertico-horizontal angle: 35 degrees inferior. Mesio-distal angle: Directly between the central incisor teeth (*fig. 6*). *Position of film packet:* With the long dimensions of the film packet corresponding to the long axes of the incisor teeth, closely adapt the packet to include equal portions of the incisor teeth. Have the upper edge of the film packet uniformly 4 to 6 mm. beyond the incisal edges of the teeth (*fig. 4*). *Retention of film packet:* The film packet is retained with the index finger in the center of the lower third, resting the thumb on the side of the mandible. *Position of cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* 3½ seconds (regular films).

General considerations: The acute angle formed by the mandible at the incisor region makes the adaptation of the film packet very difficult and distortion of the images will result unless the packet is

very carefully placed and retained. It may be helpful to use a cotton roll placed between the tissues and the film packet. The mesio-distal angulation may have to be modified to avoid the superimposition of the incisor roots upon each other.

MANDIBULAR CANINE REGION. *Position of head:* The dog is placed on his back and the head positioned so the long axes of the lower incisor teeth are horizontal. *Angulation:* Vertico-horizontal angle: 35 inferior. Mesio-distal angle: 10 degrees distally (*fig. 6*). *Position of film packet:* With the long dimension of film packet corresponding to long axes of the incisor teeth, closely adapt the packet to include the first incisor teeth and extend distally. Have the upper edge of the film packet 4 to 5 mm. above the incisal edges of the incisor teeth and 6 to 8 mm. above the cusp of the canine (*fig. 4*). *Retention of film packet:* The film packet is retained with the index finger in the lower mesial corner, resting the thumb over the side of the madible. *Position of cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* $3\frac{1}{2}$ seconds (regular films).

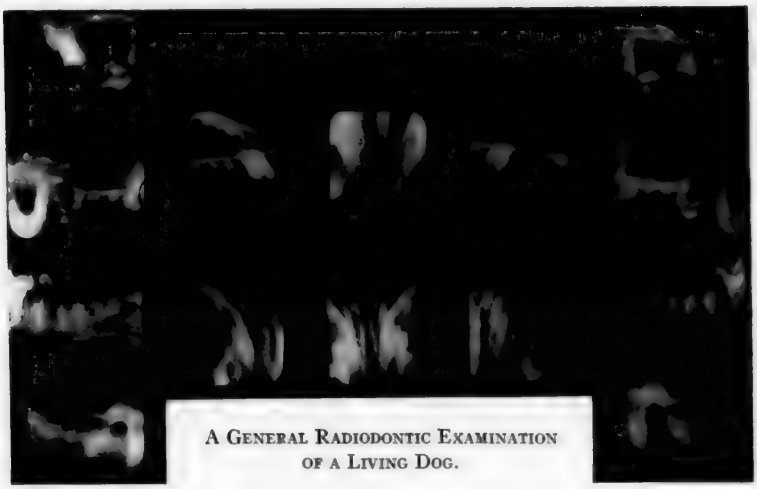
General considerations: The chief difficulty encountered in this region is securing undistorted images of the canine roots without the superimpositions of the bodies of the mandible at the symphysis. The mesio-distal angulation must be varied accordingly in such cases.

MANDIBULAR PREMOLAR REGION. *Position of head:* The dog is placed on his side and the head positioned so the side to be radiographed is horizontal. *Angulation:* Vertico-horizontal angle: 45 degrees inferior. Mesio-distal angle: Perpendicular to the film packet (*fig. 6*). *Position of film packet:* With the short dimension of film packet corresponding to long axes of the teeth, closely adapt the packet to include the first premolar and extend distally. Have the upper edge of the film packet uniformity 5 to 6 mm. above the cusps of the pre-molar teeth (*fig. 3*). *Retention of film packet:* The film packet is retained with the index finger in the lower mesial corner, resting the thumb under the canine region. *Position of cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* 4 seconds (regular films).

General considerations: It is practically impossible to avoid dis-

tortion in the extreme lower mesial corner of the film packet due to excessive bending of the packet at this point. A slight shift of the tube to the mesial will aid in preventing much of this distortion. Care must be taken to keep the tongue from interfering with the respiration of the animal.

MANDIBULAR MOLAR REGION. *Position of head:* The dog is placed on his side and the head positioned so the side to be radiographed is horizontal. *Angulation:* Vertico-horizontal angle: 75 degrees inferior. Mesio-distal angle: Perpendicular to the film packet (*fig. 6*). *Position of film packet:* With short dimension of film packet corre-



A GENERAL RADIODONTIC EXAMINATION OF A LIVING DOG.

sponding long axes of the teeth, closely adapt the packet to include all the third molar region and extend mesially. Have the upper edge of the film packet uniformly 3 to 4 mm. above the cusps of the molar teeth (*fig. 3*). *Retention of film packet:* The film packet is retained with the index finger in the center of the lower one third, resting the thumb under the pre-molar region. *Position of cone:* The cone is centered so the central rays will pass midway thru film packet. *Exposure:* 4½ seconds (regular films).

General considerations: The mandibular molar region is probably

the ideal to radiograph in that the film packet can be placed practically parallel to the long axes of the molar teeth and accurate profile images of the teeth secured. Care should be taken in placing the packet in this region so the soft tissues of the throat will not be severely traumatized. The film packet must be retained firmly in order to avoid movement of the packet during exposure.

GRENZ RAY STUDIES OF THE CALCIFICATION OF ENAMEL

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REVIEW OF LITERATURE

V. von Ebner (1906) attempted to classify the changes in enamel from the soft embryonic state to the stone-hard condition it finally reaches. On the basis of its appearance in polarized light he proposed 4 terms to describe the stages of enamel in passing from its original soft condition to the finished, hard state: primary, young, transitional and hard.

It may be advisable to discuss some terms especially since they have been used with different meanings or emphasis by many investigators. Thus, ***development*** of enamel implies the formative processes associated primarily with cellular activity of the ameloblasts. ***Calcification*** on the other hand deals with subsequent processes superposed on development or formation—namely, the deposition and precipitation of mineral salts.

The calcification of the enamel has been described by Beust (1928) as occurring in several stages. Beust demonstrated this by staining and by x-ray studies. Kitchin confirmed this by polarized light studies, while more recently Chase employed the decalcification method to show that enamel which has just been formed (organic) resists solution in acids. Later on, it is relatively acid soluble. The difference must be due to some transformation taking place after enamel has been deposited by the ameloblasts.

METHOD AND MATERIAL

The present report is based on Grenz ray (soft x-ray) studies (Applebaum, Hollander and Bodecker, 1933) of ground sections of deciduous and permanent human teeth—and briefly presents the calcification process, essentially as previous workers have described it. An attempt was made to arrange the material so as to show the stages

of enamel calcification as simply as possible. Ground sections were made as nearly uniform in thickness as possible with hand ground material. Photomicrographs and Grenz-ray pictures were taken and uniform enlargements secured. An attempt was made to secure uniformity in the voltage, amperage, and exposure time of all Grenz ray plates so that the different sections could be compared in regard to extent and degree of calcification.

FINDINGS

The beginning of calcification can be studied in *fig. 1*, a photomicrograph of an unerupted deciduous second molar of a still-born infant.

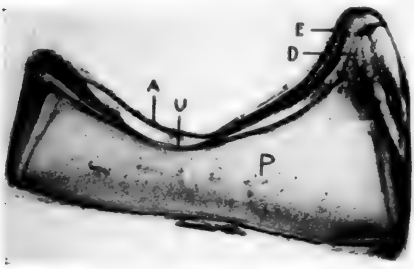

FIG. 1

FIG. 2

FIG. 1. Photomicrograph of unerupted deciduous second molar of a still-born infant (9 months). Bucco-lingual ground section showing dentine (D) and enamel formation (E) at horns of dental papilla (P). These separate centers of calcification are joined across occlusal surface by predentine (U) which is uncalcified. A is layer of ameloblasts displaced during grinding.

FIG. 2. Grenz ray (soft x-ray) picture of specimen seen in *fig. 1*. Beginning of calcification is seen at separate centers. There is a difference in density of enamel and dentine even at this early stage.

This is a bucco-lingual ground section showing enamel and dentin formation at the tip of the separated cusps, with uncalcified dentin alone uniting the cusps across the occlusal surface. The specimen was impregnated with balsam in order to retain the soft tissues during grinding, and we see the dental papilla as well as the calcified enamel and dentin. A Grenz ray picture (*fig. 2*) of this ground section shows a variation in density in the different tissues. Even at this very early stage a difference is noted between density of enamel and dentin at the tip of the cusps. The uncalcified dentin (predentin) which

serves to join these cusps is radiolucent, and we only know it is present by microscopic examination of the specimen.

Fig. 3 is a photomicrograph of a ground section of an unerupted first deciduous molar of an infant, one year and six months old. In this case the enamel crown seems completely calcified. Yet in the Grenz ray picture, *fig. 4*, we note that while the enamel along the occlusal surface is radiopaque, the enamel along the buccal and lingual plates of the crown is much more radiolucent. This shows that although the enamel is completely formed, calcification is not completed. An aluminum penetrometer was inserted in order to give

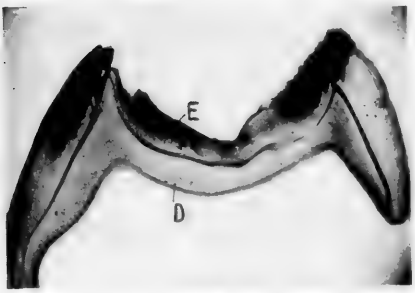

FIG. 3

FIG. 4

FIG. 3. Photomicrograph of unerupted deciduous molar from infant 1½ years old. Bucco-lingual ground section showing complete "enamel" formation. E = enamel. D = dentine. Enlargement 20×.

FIG. 4. Grenz ray (soft x-ray) picture of specimen seen in *fig. 3* indicating that enamel is not uniformly calcified though completely "formed". Note "gray scale" of aluminum penetrometer.

some idea of the relative densities of the tissues during development. Van Huysen, Warren, Hodge and Bishop, have shown that normal unchanged dentin is nearly equivalent, in its roentgen ray absorption, to an equal thickness of pure aluminum while enamel is nearly equal to twice its equivalent thickness. The gray scale of x-ray negatives from white to black can be divided into many intermediate values. These variations depend on the radiopacity of the dental tissues. They may be compared with the gray scale made by the aluminum penetrometer which has steps of different thickness marked 1, 2, 3, 4, and 5 as seen in *fig. 4*.

In comparing the shades registered by the penetrometer to those of the tissues of the tooth we know that Step 1 of the penetrometer is about twice as thick (dense) as Step 5. Occlusal enamel (white) is about the same shade as step 1 and therefore is twice as dense as the approximal enamel which is as black as step 5. The occlusal dentin is about midway between these in density or equivalent to step 3 = 280μ of aluminum, which is the thickness of this ground section.

Fig. 4 also seems to indicate delicate variations in radiopacity along the striae of Retzius. This picture seems to support the point of view of Chase concerning the rhythmicity of enamel maturation:

> "Beust (1928) has called attention to the relatively insoluble immature enamel of the pig and the fact that it is present in nearly its entire adult thickness before the hardening process, which requires months, is completed. His statement that 'that which has heretofore been taught about the *rhythmic* deposition of the enamel whereby the striæ of Retzius are said to be formed is based on a misconception' is correct in the sense of his further statement that 'the ameloblasts do not deposit finished layers of enamel' but is certainly incorrect in the sense that there is no rhythmicity in the maturation of the enamel. That maturation of the enamel is a rhythmically progressive process has already been demonstrated polariscopically by von Ebner (1906) whose findings are supported by my demonstration of the differentially staining zones in the decalcified sections of immature enamel. . . . The boundaries between young and transitional enamel parallel the direction of the incremental lines."

Fig. 4 is proof that enamel is not laid down by ameloblasts as a fully formed and calcified product. Mellanby claims that enamel calcifies *in two stages*, the tissue being first deposited in a semi-calcified state and later becoming fully calcified. If the first process is slowed down or checked, the resulting enamel will be below normal in quantity but it may still be well calcified. Thus, in spite of irregularities in enamel surface with gross defects, the enamel of the specimen shown in *fig. 5*, is radiopaque in the Grenz ray picture, *fig. 6*.

When, however, the deposition of the semi-calcified basis is complete, but the second stage of calcification interrupted, then although there may not be any deficiency of enamel it is defective in other respects. This is seen in a study of mottled enamel (Applebaum, 1936) showing radiolucent defects, in outer layers of enamel, which persist throughout life (*figs. 7–8*). So-called mottled enamel is apparently enamel which has not completed calcification, since it is often retained in its entire thickness after decalcification. In decalcifying

human mottled enamel the writer noted that the organic matrix of enamel stained blue (Mallory's stain) throughout—even at the surface. This enamel has remained in its original form and has not proceeded to the final stage of calcification.

One is tempted at this point to speculate on the mechanism of calcification. Obviously from *fig. 4* the ameloblasts do not lay down a fully formed and calcified enamel. There seems to be an organic matrix formed first which is calcified later. Wells has pointed out that we are driven to the conception of a physical *Kalkfanger* which

FIG. 5

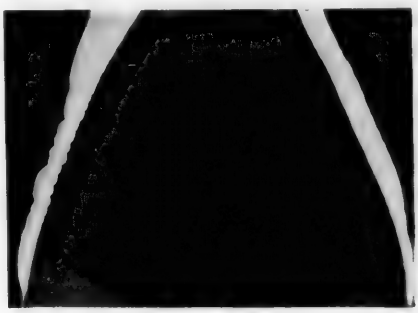

FIG. 6

FIG. 5. Hypoplastic human enamel with zones of injury following striae of Retzius. Note interglobular spaces in dentin at I.

FIG. 6. Grenz ray picture of hypoplastic enamel seen in *fig. 5*. Note poorly calcified interglobular spaces in dentine.

serves as a point where condensation of poorly soluble calcium salts takes place until, perhaps because of variation in CO_2 content in the solvent fluids (blood and lymph), precipitation begins and continues rhythmically as Hofmeister suggested. Wells showed that the substances which take up calcium have strong and specific absorption affinities. This is interesting in relation to *fig. 2* where at the beginning of calcification we note a difference in the density of enamel and dentin. Presumably the enamel and dentin matrices have different absorption affinities for calcium.

In the next case, *fig. 9*, various points are shown. We see an ordinary clinical x-ray of the anterior portion of the mandible of a child, one year old. This specimen was sawed through at the median

FIG. 7

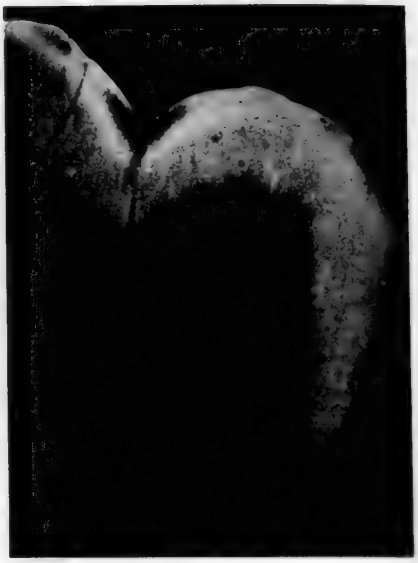

FIG. 8

FIG. 7. Photomicrograph of ground section of impacted mottled lower molar. (Dr. Martin's case—Aberdeen, South Dakota.) (Some of surface enamel was lost in grinding due to great brittleness of tissue.)

FIG. 8. Grenz ray picture of impacted mottled molar seen in *fig. 7*. The x-ray negative indicates variations in density of enamel along bands of Schreger at outer third of enamel. This defect in calcification at enamel surface is no denser than dentine in this thin section.

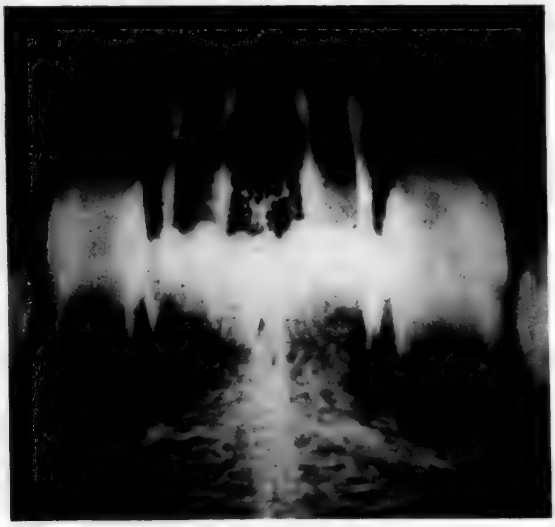

FIG. 9. "Clinical" x-ray picture of incisor region of year old child's mandible. Note fully erupted deciduous incisors and unerupted permanent incisors.

line. The right half was ground and the left half was decalcified. When Grenz ray studies (*fig. 11*) were made of the ground section (*fig. 10*) certain details were noted concerning calcification which were not apparent in the hard x-ray (clinical) picture commonly used. The degree of calcification or radiopacity of the enamel in the ground sections (*fig. 10*) is nicely correlated with the degree of acid solubility

FIG. 10

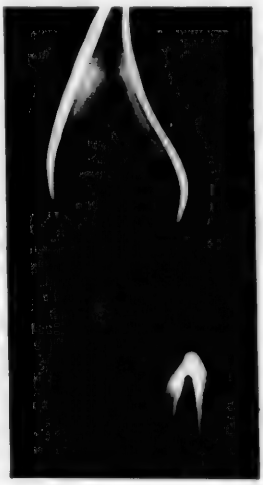

FIG. 11

FIG. 12

FIG. 10. Photomicrograph of fully erupted lower *right* deciduous incisor and unerupted permanent incisor. Labio-lingual ground section of case shown in *fig. 9*.

FIG. 11. Grenz ray picture of ground section shown in *fig. 10*. Enamel of erupted incisor is uniformly calcified while that of unerupted tooth is calcified at incisal tip and dento-enamel junction.

FIG. 12. Photomicrograph of fully erupted lower *left* deciduous incisor and unerupted permanent incisor. Decalcified labio-lingual section of case shown in *fig. 9*. (Note organic matrix of enamel in unerupted tooth.)

which is seen in the corresponding decalcified section (*fig. 12*). Thus the enamel of the erupted deciduous right incisor (*fig. 10*) is seen to be uniformly dense in the Grenz ray picture (*fig. 11*). The enamel of the corresponding left deciduous incisor (*fig. 12*) is completely dissolved in the decalcified section. The enamel of the unerupted permanent right incisor (*fig. 10*) is seen to vary in its density of calcification in the Grenz ray picture (*fig. 11*). The enamel at the tip and

dentino-enamel junction of this tooth is radiopaque and the labial and lingual plates are radiolucent. The enamel at the incisal tip of the corresponding left permanent incisor (*fig. 12*) is dissolved in the decalcified specimen while the labial and lingual plates remain insoluble.

DISCUSSION

By correlating *figs. 10*, *11*, and *12* it seems permissible to classify von Ebner's first 3 stages of enamel calcification (primary, young and transitional) as one stage since they are all largely organic, insoluble in acid and, as we have shown, radiolucent. The final stage of hard enamel is radiopaque, largely inorganic and soluble in acid.

These studies confirm Chase's demonstration in decalcifying and staining enamel that the enamel undergoes changes after it is first laid down. The stages (following von Ebner) are primary, young, transitional, and "mature" or hard enamel. The first 3 stages are insoluble in acid and radiolucent. Completely acid-soluble enamel is "mature," hard and radiopaque.

In a personal communication, Schour wrote that these Grenz ray findings "offer valuable clarification on the question of what interpretation we may be permitted to give to decalcified sections stained with hematoxylin and eosin. Your findings definitely show that the predentin which takes eosin in decalcified sections is radiolucent in Grenz rays and that the organic enamel matrix which takes on deep hematoxylin is also radiolucent in the Grenz ray. This does not mean merely that there is no calcium in the eosin stained predentin or in hematoxylin stained organic enamel matrix. Calcium may be present but merely in organic form and the organic calcium compound apparently does not resist the Grenz ray."

Chase claimed that after the "maturation" of enamel, which in the human dentition, takes approximately 3 months, "there is no further change that affects its solubility during technical processes of decalcification." Evidently in his paper Chase uses the term maturation to apply to calcific changes in enamel prior to eruption. However, in a personal communication, Chase wrote that he would not make the positive statement that "there are no post-eruptive changes in human enamel. As a matter of fact there is considerable evidence

that enamel does become altered with age, for example, it is generally believed to become more brittle."

Beust's (1934) concept of tooth maturation applies to "all parts of the tooth, its progress depending on the degree of reduction of the pulp chamber which finally may become entirely obliterated in the mature tooth. . . . In the fully matured tooth all colorable (chromphilic) tissue has disappeared. . . . During maturation a hypercalcified (possibly also keratinized) tooth replaces a young organ with a high organic content."

Karlstrom has made a great many tests of the hardness of dental enamel by means of the Herbert Pendulum Hardness Tester. He showed that the inner zone of enamel in erupted fully mature teeth was harder than that of newly erupted teeth. But, as the surface hardness of *immature* and *mature* enamel is nearly the same, he comes to the conclusion that the mineral salts for the posteruptive calcification of the inner zones of the enamel originate from the interior (dental pulp) and not from the exterior (salivary salts).

Hollander has also investigated the question of post-eruption enamel changes. He employed a precision method for studying the radiopacity of ground sections and found that enamel contains nearly twice as much calcium salt per unit volume as does the dentine. Hollander concluded from a preliminary study of 4 teeth from different individuals, 17 to 68 years old, that "there exists a saturation value for the normal deposition of calcium salts in each region of the tooth and although any particular area may be undersaturated, a condition of oversaturation does not normally occur." Hollander, in employing soft x-rays, pointed out that the usual mode of chemical analysis is not always capable of detecting progressive changes in calcification of an organic matrix.

It may well be that certain subtle changes are studied better by one technic than by another. Thus changes in the character of a mineral have been ascribed to molecular rearrangement without addition of any new material, e.g. calcite to aragonite—which is the stable form of calcium carbonate. Such changes would not be apparent in chemical analysis, yet easily observed by polarized light. Similarly, certain posteruptive changes in enamel might not be disclosed using Chase's method of acid solubility as the sole test. But changes may occur

in the enamel which can only be disclosed by the hardness or staining tests described by Karlstrom and Beust. Such changes may be the cause of increased translucency observed in enamel after eruption.

SUMMARY

An attempt was made to clarify the mechanism of enamel calcification. The old concept that enamel is laid down as a fully formed and calcified product is refuted by means of soft x-ray pictures. While the enamel is apposed in incremental layers its calcification is shown to pass through different stages en masse, confirming the work of von Ebner, Beust, Chase, etc.

One stage (under which the primary, young and transitional stages of von Ebner, are grouped) is radiolucent and insoluble in acid. A final stage is soluble in acid and radiopaque at 10,000 volts. The question of post-eruptive changes in enamel is discussed.

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STUDIES ON THE CHEMISTRY OF MIXED HUMAN SALIVA

III. The Composition of Saliva Produced at Varying Rates of Secretion by the Same Person with Special Reference to Calcium and Phosphorus

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In previous reports (1) (2) the composition of mixed human saliva was studied in groups of individuals who naturally produced saliva at widely varying rates of secretion. In the present investigation a large number of salivary specimens was obtained from 2 men under a variety of experimental conditions. In the first section of this report we have studied the content of calcium and phosphorus in a number of 1 hour specimens obtained from 1 donor. In the second section salivary specimens were obtained from another donor under such varying conditions that he produced from 28 to 213 cc. per hour. In the third section are described specimens from 1 person during a continuous 6 hour chewing period.

METHOD

The methods used in the chemical work below were essentially those reported previously (1) (2). We have modified considerably, however, the procedure for calcium and phosphorus, especially as regards preparation of the sample for analysis. The details of the procedure are as follows: Measure 10 cc. of saliva into a 40 cc. platinum dish. Evaporate to dryness on a hot plate. Ash at 650°C in a muffle furnace, equipped with a pyrometer. Dissolve the ash in exactly 10 cc. of 0.1 N. HCl, triturating with a small glass rod to facilitate solution.

Phosphorus was determined in this solution by the colorimetric procedure of Youngburg (3). Calcium was determined on 2 cc. aliquots by precipitation with ammonium oxalate. The pH was adjusted to 4.5 with 0.05 N $\cdot NH_4OH$, using methyl red as an indicator. The remaining details were essentially those of the Clark-Collip modification of the Kramer-Tisdall method (4), except that the calcium oxalate was washed with dilute ammonia saturated with calcium oxalate.

The question of whether to use centrifuged or uncentrifuged samples of saliva was very troublesome. The most comprehensive discussion of this problem has been given by Wainwright (5) who reported that in 6 of 10 specimens examined no loss of calcium occurred. In the other 4 the results were irregular. Wainwright concluded, that on

account of this irregularity, centrifugation should precede analysis. In our own experience centrifugation has little effect on the phosphorus content, but almost invariably the calcium is lowered 1–2 mg./100 cc. One of our reasons for deciding against centrifugation in this series of experiments was that our specimens were stimulated salivas produced

TABLE I

Analyses of salivas of H. W.

	SAMPLE NO.	VOLUME 15 MIN.	TOTAL SOLIDS	ASH	Ca	P	P/Ca
		cc.	%	%	mg./100 cc.	mg./100 cc.	mg./100 cc.
Experiment (1)	1	32	0.766	0.224	7.30	13.5	1.84
6/14/35, 10–11	2	27	0.660	0.190	7.00	14.3	2.04
A.M.	3	33	0.672	0.254	6.60	11.9	1.80
	4	21	0.735	0.265	5.55	12.6	2.27
		Total 113	Avg. 0.683	0.233	6.61	13.0	1.98
Experiment (2)	1	33	0.672	0.226	6.55	12.2	1.86
6/14/35, 2–3	2	33	0.618	0.202	7.35	10.3	1.40
P.M.	3	31	0.512	0.216	5.35	14.8	2.76
	4	33	0.728	0.252	5.30	16.0	3.01
		Total 130	Avg. .0632	0.224	6.13	13.3	2.25
Experiment (3)	1	27	0.802	0.210	7.65	17.3	2.26
6/19/35 9–10	2	20	0.590	0.124	7.85	15.7	2.00
A.M.	3	28	0.582	0.218	6.70	14.1	2.10
	4	29	0.578	0.224	5.60	14.0	2.50
		Total 104	Avg. 0.635	0.194	6.95	15.2	2.21
Experiment (4)	1	27	0.643	0.225	6.05	15.6	2.57
6/19/35, 2–3	2	36	0.656	0.250	6.25	14.7	2.35
P.M.	3	30	0.556	0.102	6.60	15.4	2.33
	4	33	0.560	0.186	5.90	15.4	2.60
		Total 126	Avg. 0.603	0.190	6.20	15.2	2.46
Experiment (5)	1	30	0.782	0.148	7.22	17.2	2.42
6/20/35, 2–3	2	23	0.786	0.116	7.22	16.5	2.28
P.M.	3	25	0.722	0.222	6.55	16.6	2.53
	4	26	0.740	0.278	6.20	17.2	2.77
		Total 104	Avg. 0.757	0.191	6.79	16.9	2.50

over 1 to 6 hour periods. The effect of food residues is thus correspondingly less because these are spread over larger volumes. Further, we are inclined to the belief that much of the precipitate which is removed from production salivas by centrifugation consists (1) of serous and mucous débris and desquamated cells of the glands which may well represent

sources of calcium which should be *accounted* for, and (2) of insoluble compounds which form gradually on standing. Usually, for example, further precipitation occurs after the specimen has stood for a day or two. Unless otherwise specified, the specimens below were uncentrifuged.

EXPERIMENTS AND OBSERVATIONS

Phosphorus and Calcium in Saliva of H. W. Obtained During 1 Hour Production Periods. In the following experiments H. W. chewed 8 gm. of paraffin for 1 hour periods. Fifteen minute specimens were collected and analysed, the results appearing in Table I.

The average production by H. D. W. on 8 gm. of paraffin was 115 cc. per hour. Apparently over 1 hour periods of stimulation calcium values tend to decline; those for phosphorus are relatively constant in 3 experiments, in 1 instance there being a rise and in another a fall. The P/Ca values fluctuate but tend to increase as stimulation continues, due to the calcium decline.

The Composition of Salivas Produced at Widely Varying Rates by H. L. One subject (H. L.) produced 1 hour specimens of saliva under various conditions of stimulation; from the mere act of moving the jaws to several other chewing stimuli,—gum, various amounts of paraffin and the like. In several instances the chewing was continued over 2 and 3 hour periods. In Table II a large series of data is arranged *according to the rate of production.* It is to be noted that the data naturally fall into 3 groups, comparable to the data in previous reports (1) (2). Averages for the 3 groups, low, medium, high, are shown for comparison as groups.

The data above lead to the general conclusion that an individual may be made to produce saliva over a wide range of production rates by simply varying the chewing stimulus. This result of course might have been predicted, although it was not expected that the range would be so wide, in fact as wide as one observes over a large group of people with the same chewing stimulus (cf. preceding reports). Chemical analyses of the large number of specimens reported above support the idea that when a given individual produces salivas at widely varying rates, the composition varies essentially as noted in our previous work on specimens produced by a large group of persons, —namely, solids, ash, chloride, and pH increase with rise in production rate; calcium rises slightly; phosphorus remains essentially con-

TABLE II

Analysis of salivas produced by H. L. under various chewing stimuli

EXPERIMENTAL CONDITIONS	CHEWING STIMULUS	VOL./HR.	TOT. % SOLIDS	ASH %	Cl	Ca	P	pH
		cc.			*mg./100 cc.*	*mg./100 cc.*	*mg./100 cc.*	
9:30 A.M.	Nothing	28	0.4830	0.1760	50.8	—	—	7.1
9:30 P.M. In bed	Nothing	29	0.5255	0.1310	42.1	—	—	7.0
6:30 A.M. Before breakfast	Nothing	32	0.5500	0.2650	56.5	—	—	—
10:45 P.M. Cold	Nothing	35	0.5730	0.2200	42.4	—	—	—
3:50 A.M. Water before	Nothing	38	0.4730	0.2190	43.0	4.0	12.6	—
10:45 P.M.	O. K.	49	0.5535	0.2090	19.5	—	—	6.8
10:45 P.M.	Nothing	51	0.5195	0.1695	37.9	—	—	7.0
9.10 P.M.	Nothing	51	0.5065	0.2770	28.7	—	—	7.0
11:40 P.M. Sore throat	Nothing	56	0.4890	0.2090	41.1	6.4	10.5	—
Low Producing, Average.....		42	0.5099	0.2028	40.2	5.2	11.5	7.0
3:10 P.M.	1 Stick Gum	80	0.4500	0.2160	37.0	5.9	14.1	7.0
2:45 P.M. (2nd hr.)	2g. P.	81	0.5060	0.2360	61.6	—	—	7.3
2:50 P.M.	1g. P.	85	0.5020	0.2150	62.3	—	—	7.1
2:00 P.M.	1g. P.	85	0.5095	0.1995	54.3	—	—	7.3
1:05 P.M.	1g. P.	89	0.4655	0.2165	—	—	—	7.3
3:50 P.M. (2nd hr.)	1g. P.	91	0.5180	0.2250	63.3	—	—	7.4
9:05 A.M.	1g. P.	97	0.4875	0.1905	81.9	5.9	12.0	7.3
4:00 P.M. (3rd hr.)	2g. P.	100	0.4570	0.2150	59.7	—	—	7.3
9:03 A.M.	R. Stopper	107	—	—	66.7	—	—	7.2
10:10 A.M. (2nd hr.)	2g. P.	112	0.4806	0.2350	77.4	—	—	7.3
10:40 A.M. (2nd hr.)	3g. P.	113	0.5015	0.2465	73.4	—	—	7.2
1:45 P.M.	2g. P.	115	0.5260	0.2630	75.2	—	—	7.3
10:27 P.M.	1 Stick Gum	115	0.5600	0.2730	58.7	5.1	11.7	6.1
10:10 A.M.	2g. P.	118	0.5255	0.2410	88.7	—	—	7.3
9:40 A.M.	3g. P.	120	0.5220	0.2260	87.8	—	—	7.3
10:53 A.M. (2nd hr.)	4g. P.	124	0.4570	0.2580	82.9	5.6	11.6	7.5
9:00 A.M.	4g. P.	126	0.4465	0.2840	92.9	4.9	11.7	7.3
12:48 A.M. Cold	2g. P.	134	0.5400	0.3770	125.5	—	—	7.4
12:35 P.M.	4g. P.	143	0.5130	0.3090	98.9	4.0	12.5	7.3
Medium Producing, Average.....		107	0.5012	0.2403	73.8	5.2	12.3	7.2
1:27 P.M.	8g. P.	173	0.5645	0.2910	83.8	6.5	12.0	7.5
4:30 P.M.	12 g. P.	189	0.6545	0.2970	96.2	7.4	11.6	—
1:24 P.M.	3 Sticks Gum	196	—	0.2900	75.3	—	—	—
9:45 A.M.	8g. P.	198	0.5550	0.2890	95.6	6.7	11.3	7.3
10:50 A.M.	8g. P.	200	0.5590	0.3040	100.6	4.6	10.9	7.3
4:30 P.M. Cold	8g. P.	213	0.6090	0.2940	88.5	6.5	11.7	—
High Producing, Average.....		195	0.5880	0.2925	90.0	6.3	11.5	7.4

stant. In general the rate of production rises with increase in amount of paraffin chewed. The increase in pH may involve increase in concentration of bicarbonate although we made no analyses for this component to test this possibility.

The Changes in Composition of Saliva Produced Continuously Over A Six Hour Period. This experiment was conducted primarily to satisfy our curiosity as to what would happen to secretion rate and composition of salivas produced during a prolonged period of chewing stimulation. One of us (H. L.) 3 hours after a breakfast of 1 orange and immediately after a lunch of 2 oranges began chewing (12 M) four grams of paraffin. Continuous hourly specimens were secured for 6 hours. About the fourth hour the subject became thirsty and remained so during the rest of the experiment, due, no doubt to dehydration. From the fifth hour on, severe headache was experienced n the occipital region. The data on the 6 specimens follow:

TABLE III

Analytical data on salivas produced continuously over a six hour period

HOUR	AMT. SECRETED	TOTAL SOLIDS	ASH	ORGANIC*	Cl	Ca	P
	cc./hr.	*per cent*	*per cent*	*per cent*	*mg./100 cc.*	*mg./100 cc.*	*mg./100 cc.*
1	155	0.518	0.301	0.217	91.1	5.8	8.7
2	129	0.475	0.279	0.196	90.4	5.6	12.4
3	102	0.495	0.241	0.254	72.6	10.0	11.4
4	107	0.412	0.232	0.180	60.7	12.1	12.2
5	87	0.383	0.220	0.163	57.9	6.1	9.4
6	75	0.387	0.247	0.140	56.5	10.6	9.3

* Organic calculated by subtracting ash from total solids.

Generally speaking the data above describe the changes in com position such as might be expected to obtain under conditions of progressively fatiguing salivary glands. The rate of secretion declines steadily. With this decline solids, ash and chloride decrease at a fairly regular rate. Data for the last 2 hours tend to be more constant. Organic matter changes irregularly with an appreciable decline towards the end. Phosphorus rises definitely; then falls back to the original level. Calcium almost doubles during the third, fourth and sixth hours. There is a general tendency apparently for the concentration of calcium during prolonged activity of the glands to

approach its concentration in the blood, namely 10–14 mg. per cent. These results, and many of our others, are not in agreement with those of Jonsgar (6) who reports lowered calcium values for "stimulated" salivas.

SUMMARY

(1) In 1 subject specimens of saliva were produced over 5 one hour periods, the specimens in each case being taken at 15 minute intervals and subjected to analysis. During the 1 hour periods there was a tendency for calcium to decline and for phosphorus to be variable but the P/Ca values showed some tendency to increase due to fall of the calcium values. (2) Another subject produced 34 one hour specimens under a wide variety of chewing stimuli. The rate of secretion varied from 28 to 213 cc. per hour. When arranged according to rate of production, the specimens fall naturally into 3 groups—low, medium and high; the average composition of these groups may be correlated with rate of production very like the results previously reported for *groups* of individuals. (3) One subject chewed continuously for 6 hours. The regular fall in production rate was accompanied by decline in solids, ash and chloride. Phosphorus rose, then fell. Calcium nearly doubled in three of the specimens, concentration approaching that of blood serum.

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HUMAN SALIVA

III. Critical Discussion of Former Salivary Phosphorus Studies and Their Value in the Establishment of Normal Standards[1,2]

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INTRODUCTION

In a recent publication (11) an attempt was made to establish the present status of technical procedures in salivary calcium analysis. Emphasis was placed on the necessity of establishing normal ranges for salivary constituents, which, based on a large amount of material, will serve as standards by which any pathologic composition can be judged. Today in any discussion of normal and pathologic calcium metabolism, due consideration must be given to the various phosphorus fractions and their distribution because it is increasingly apparent that they are interdependent in metabolic processes. For many years it was believed that dental caries, paradentosis and pathologic bone formation were primarily calcium problems, because one is easily inclined in speaking of enamel, dentin, cementum and bone to think in terms of only their chief constituent. However, recent authors [McCollum (84); Klein and McCollum (63, 64, 65); Agnew, Agnew and Tisdall (4, 5); and Price (94, 95, 96, 97, 98, 99, 100)] have expressed the suspicion that caries might be the manifestation of a disturbed phosphorus metabolism which reveals itself in the salivary

[1] Conducted under grants made by the Carnegie Corporation, the California State Dental Association, California Academy of Periodontology, Research Board of the University of California and the Good Teeth Council for Children Inc.

[2] Presented at a joint meeting of the San Francisco Section of the International Association for Dental Research and the Northern California Section of the American College of Dentists in San Francisco, March 10, 1937.

composition. Here, as with calcium metabolism, there is a profuse literature on the subject of pathologic levels of salivary phosphorus without any consideration of comparative values of normal composition. The establishment of standard norms must at present be considered the primary requisite for the study of pathologic material.

Earlier analyses of inorganic salivary phosphorus (10) focused attention on the wide range of values found in various individuals. It is surprising that inorganic phosphorus values of mixed human saliva range between 10 and 70 milligrams per cent, in contrast to those of blood, which range between 3 and 5 milligrams per cent. Such a range is unequaled in any other secretory product of the body and it brings to mind a problem which has been discussed frequently—whether the saliva is solely a *secretory* product or whether it acts more or less as a "safety valve" for the organism and thereby becomes also a typical *excretory* product. This involves the question of (a) whether the phosphorus composition of saliva can be influenced by diet or the administration of drugs in a typical manner and (b) whether the organism controls the phosphorus output through the salivary glands in a similar way as through feces and urine.

In view of the fact that the phosphorus intake varies individually to a large extent, it is, of course, possible that each individual food habit might be reflected in the saliva. Extreme variations, therefore, may have definite biological significance and it was felt that parallel with investigations on salivary calcium the various phosphorus fractions should be examined. Before analytical work was begun the literature was surveyed as to formerly established averages and ranges for salivary phosphorus.

APPROACH OF FORMER INVESTIGATORS IN THE ANALYSIS OF SALIVARY PHOSPHORUS AND CRITICAL DISCUSSION OF THEIR WORK

Salivary phosphorus analyses have been made as far back as 1848 by Jacubovitch (54). References to his analyses can be found in Bidder and Schmidt's work on saliva in 1852 (15). During the following decades the names of Hammerbacher, 1881 (42), Herter (49), Frerichs (38), Enderlin, 1884 (28) and Jacobi, 1896 (53) are definitely connected with the history of analysis of mixed human saliva. They all used macro methods in determining the phosphorus in the form of

phosphorus pentoxide (P_2O_5) or tertiary calcium phosphate ($Ca_3(PO_4)_2$) from ash analysis and expressed this in the form of percentage of ash. Even as late as 1912 we find reference to analytical work by Evans (31), who used a macro method for 11 cases. Development of the more delicate micro methods in use today, which permit the analysis of very small individual samples, has led to the belief that these early results can hardly be considered for comparative purposes.

An endeavor to evaluate the work of former investigators of salivary phosphorus, with the omission of the earlier ones mentioned above, encountered considerable difficulties. A number of authors did not give adequate information as to their analytical procedure, making it impossible to classify them and to estimate the comparative value of their results (104, 66, 45, 46, 47, 48 and 26). They omitted the most essential data, not even mentioning the fraction analyzed.

For the sake of completeness their names and available data are entered in Table I; however, they are grouped separately from those authors who are classified according to the fraction analyzed (total phosphorus, total acid soluble and inorganic phosphorus).[3]

Group I. Total Phosphorus. Among 44 investigators of salivary phosphorus the authors found references to eighteen who paid special attention to the total phosphorus content (Table I). Röse's (102) analyses of pooled samples belong to the earliest investigations of total phosphorus of saliva. However, no reference could be found as to the technic followed in his work. The data published by Price in 1932 (96) and 1936 (101) are also most incomplete and make it difficult to interpret the vast amount of material examined. Between 1905 and 1927 apparently no reports appeared in the literature with reference to salivary total phosphorus until Clark and Levine (20) again opened the discussion of this problem in 1927. Since then it seems that investigators have become more "phosphorus conscious."

The importance of preliminary centrifugation for the removal of debris, etc. in the technic of phosphorus analysis will be discussed separately (115). However, it seems worthy of note that of the 18 investigators, only 2 used preliminary centrifugation of saliva (19, 117); 2 did not state which procedure they followed (102, 96); while

[3] The report of the 225 cases of Vogt (113) referred to by Enright, Friesell and Trescher (29, page 795) for calcium and phosphorus analysis of saliva was not available.

TABLE I

Details of technical procedures of the various fractions used by former investigators

1 PHOSPHORUS FRACTION	2 DATA INCOMPLETE	3 AUTHOR	4 YEAR	5 PRELIM. CENT.	6 SALIVA FOR SINGLE DETERM.	7 PRELIMINARY TREATMENT OF ORGANIC MATTER	8 METHOD Name	9 METHOD Type	10 METHOD CHECKED ON TEST SOLUTION	11 COMMENT
					cc..					
Not reported	•	Ruthrauff	1923	?	?	?	?	?	Not reported	
	•	Knoche	1927	?	?	?	?	?	Not reported	
	•	Hawkins	1931	?	?	?	"Bell-Doisy-Briggs"	Colorimetric	Not reported	
	•	Eddy, Heft, Rosenstock and Ralston	1933	?	?	?	Fiske and Subbarow	Colorimetric	Not reported	
I. Total phosphorus	•	Rõse	1905	?	? Pooled samples	Dry ash	?	?	Not reported	Rõse reports in one case 42 samples were pooled giving an amount of 1,623 cc.
		Clark and Levine	1927	No	?	Dry ash	Benedict-Theis	Colorimetric	Not reported	
		Karlström	1927	No	?	Dry ash	Hawk	Gravimetric	Not reported	
		Roskin	1927	No	?	Dry ash	Benedict-Theis	Colorimetric	Not reported	
	•	Entin and Geikin	1928	No	—	Dry ash, see comment	Samson	Volumetric	Not reported	Entin and Geikin determined "total P" by adding organic acid insoluble and inorganic fractions
		Hubbell and Bunting	1932	No	0.4–0.8	Dry ash	Fiske and Subbarow	Colorimetric	Not reported	
		Karshan, Krasnow and Krejci	1932	No	0.4	Dry ash	Tisdall, Fiske and Subbarow	Colorimetric	Yes	

I. Total phosphorus	•	Price	1932	?	?	?	Kutner and Cohen	Colorimetric	Not reported	
		Youngburg	1932	No	0.1	Wet ash	Youngburg	Colorimetric	Yes	
		Adams and Myers	1933	No	?	Wet ash	Fiske and Subbarow	Colorimetric	Yes	Saliva filtered as preliminary treatment
		Boyd, Drain and Stearns	1933	No	?	Dry ash	Benedict-Theis	Colorimetric	Not reported	
		Hubbell	1933	No	?	Dry ash	Fiske and Subbarow	Colorimetric	Not reported	
		Brown and Klotz	1934	Yes	?	Dry ash	Youngburg	Colorimetric	Not reported	
		Koehne, Bunting and Morrell	1934	No	?	Dry ash	Fiske and Subbarow	Colorimetric	Not reported	
	•	Mathis	1934	No	?	?	H. Lieb	Microgravimetric	No	
	•	Price	1936	?	?	?	Kutner and Cohen	Colorimetric	Not reported	
		White and Bunting	1936	Yes	?	Dry ash	Fiske and Subbarow	Colorimetric	Not reported	
	•	Lische	?	?	?	?	?	Gravimetric	?	
II. Total acid-soluble phosphorus		Clark and Shell	1927	No	?	Deproteinization, wet ashed	Bell-Doisy	Colorimetric	Not reported	CCl_3Ac deproteinization
		Smith	1930	No	1.7	Deproteinization, wet ashed	Modified Fiske and Subbarow	Colorimetric	Not reported	CCl_3Ac deproteinization; preliminary precipitation as $MgNH_4PO_4$
		Weber	1930	No	1.0	Deproteinization, wet ashed	Modified Fiske and Subbarow	Colorimetric	Not reported	CCl_3Ac deproteinization
		Youngburg	1932	No	0.1	Deproteinization, wet ashed	Youngburg	Colorimetric	Yes	CCl_3Ac deproteinization
	•	Mathis	1934	No	?	?	H. Lieb	Microgravimetric	No	

TABLE I—*Concluded*

1 PHOSPHORUS FRACTION	2 DATA INCOMPLETE	3 AUTHOR	4 YEAR	5 PRELIM. CENT.	6 SALIVA FOR SINGLE DETERM.	7 PRELIMINARY TREATMENT OF ORGANIC MATTER	8 METHOD Name	9 METHOD Type	10 METHOD CHECKED ON TEST SOLUTION	11 COMMENT
					c.c.					
	*	Pickerill	1923	?	?	None	Pincus	Volumetric	Not reported	
		Simons	1925	No	15–25	None	Pincus	Volumetric	Not reported	
		Clark and Shell	1927	No	?	Trichloracetic acid deproteinization	Bell-Doisy	Colorimetric	Not reported	
		Entin and Gelkin	1928	No	0.5	Trichloracetic acid deproteinization	Samson	Volumetric	Not reported	
		Becks	1929	Yes	0.8	Trichloracetic acid deproteinization	Bell-Doisy, Fiske and Subbarow	Colorimetric	Not reported	
	*	Horton, Marrack and Price	1929	?	?	?	?	?	Not reported	
III. Inorganic phosphorus		Smith	1930	No	6.7	Trichloracetic acid deproteinization	Modified Fiske and Subbarow	Colorimetric	Yes	
		Weber	1930	No	1	Trichloracetic acid deproteinization	Modified Fiske and Subbarow	Colorimetric	Not reported	
		Karshan, Krasnow, and Krejci	1931	No	0.4	Trichloracetic acid deproteinization	Tisdall	Colorimetric	Yes	
		Axmacher	1932	No	?	Trichloracetic acid deproteinization	Fiske and Subbarow	Colorimetric	Not reported	
		Forbes	1932	No	0.4	Trichloracetic acid deproteinization	Fiske and Subbarow	Colorimetric	Not reported	
		Forbes and Gurley	1932	No	0.4	Trichloracetic acid deproteinization	Fiske and Subbarow	Colorimetric	Not reported	
		Krasnow, Karshan and Krejci	1932	No	0.4	Trichloracetic acid deproteinization	Tisdall, Fiske and Subbarow	Colorimetric	Yes	

III. Inorganic phosphorus	Krasnow	1932	No	0.4	Trichloracetic acid deproteinization	Tisdall	Colorimetric	Yes	
	• Price	1932	?	?	?	Kuttner and Cohen	Colorimetric	Not reported	
	Youngburg	1932	No	0.1	Trichloracetic acid deproteinization	Youngburg	Colorimetric	Yes	
	Mathis	1934	No	1.25	Trichloracetic acid deproteinization	?	Colorimetric	No	
	• Price	1936	?	?	?	Kuttner and Cohen	Colorimetric	Not reported	

12 omitted centrifugation before ashing. The prevailing method of treating organic matter (Table I, column 7) was the dry ashing process, with the exception of 2 (118, 1) who used wet ashing. The colorimetric procedure was preferred by most authors (Table I, column 9). Again, as in the reports of calcium analysis, it was surprising to learn that only 3 authors reported definitely that they checked their method on test solutions (71, 118, 119 and 1).

In Table II, columns 10 and 11, the ranges and average values obtained by these 18 authors are recorded.[4] The great variation of results becomes immediately evident. Since this variation could not be explained only on the basis of the number of patients examined (Table II, column 4), it was necessary to analyze these results (a) from the standpoint of whether resting or activated saliva was used (Table II, column 5) and (b) whether or not patients of different age groups (Table II, column 8) or states of health (Table II, column 9) were examined. The influence of variations in the analytical procedure will receive special consideration later. In a previous communication (11) the terms "resting" and "stimulated" saliva were used. "Resting saliva" referred to the sample taken before breakfast. The influence of a meal or any other stimulant, applied intentionally or unintentionally, on samples taken 1 or 2 hours after breakfast or luncheon does not permit the designation "resting saliva." It has been repeatedly stated in various publications that saliva was taken "after breakfast," or simply that "activated saliva" was used, without giving the exact time of day at which it was procured. It has been our experience that the rate of flow of saliva influences definitely the levels of calcium and phosphorus in milligrams per cent and milligrams per hour, hence it is necessary to know which type of resting or activated saliva was used and whether the rate of flow was observed and recorded.

It has been emphasized previously that immunity to caries is not an indicator of the health of the patient and that physical examinations and metabolic tests are required to be sure that otherwise unnoticed general disturbances have not influenced the results.

The data of Table II have been demonstrated graphically in Table III.

[4] The *comparative percentage* distribution of the phosphorus fractions, including the *organic* matter will be published separately.

In section A the range and averages are entered opposite the authors' names, the number in brackets next to "R" or "S" over the "O" line indicate the number of individuals examined and whether resting or stimulated saliva was used. Only 5 of these 18 authors used resting saliva (section B). With regard to the age of the patient, only White and Bunting (117) (Table III, section C) paid attention to this factor. They, too, reported their technic and material adequately (Table III, section D). However, it should be mentioned that White and Bunting described their material as "caries-free school children," without giving any other details as to their physical health.

The values for total phosphorus (ranging from 18.1 to 21.0 milligrams per gram) as reported by Scoz (106) have not been entered in Tables II and III. Although this author refers to his use of the Lohman method for phosphorus analysis of biological material, he does not state which method was used for determination of salivary phosphorus. Statements as to methods of preliminary treatment by which the various fractions were determined were omitted by Scoz and the units of measurement differ from those in general use. For these reasons it is impossible to compare his results with those of other authors.

The only range for total phosphorus content of saliva which seems to be acceptable for the establishment of a normal standard is that reported by White and Bunting: 14.1 to 18.1 milligrams per cent (117).

This unexpected inadequacy of the reports on biochemical analysis of saliva, especially with reference to total phosphorus content, demonstrates clearly that too little is definitely known on this subject.

Group II. Total Acid-Soluble Phosphorus. The total acid-soluble fraction was examined by 5 investigators (Tables I and II). Mathis (82) and Youngburg (118) included this fraction as part of their study of all fractions. Weber (116) and Smith (110) used it in their studies of the presence of organic phosphorus and its variation under different conditions of bacterial and enzyme activity. Clark and Shell (21) were concerned only with the fact that they could secure comparable results by the omission of wet ashing and chose to run the main part of their experiment directly on the deproteinized filtrate. Thus the results in this group were used only for comparison

TABLE II

Material and results of salivary phosphorus analysis used as standards for comparison by former investigators

1 PHOSPHORUS FRACTION	2 DATA INCOMPLETE	3 AUTHOR	4 NO. OF CASES	5 RESTING OR ACTIVATED SALIVA	6 BEFORE OR AFTER BREAK.	7 RATE OF FLOW	8 AGE OF PATIENTS	9 HEALTH OF PATIENTS	10 RESULTS Range	11 RESULTS Av.	12 ADDITIONAL MATERIAL AND REMARKS
									mg. %	*mg. %*	
Not reported	•	See table 1	Data omitted here because authors failed to report which phosphorus fraction they analysed; essential data of technical procedure and type of clinical material used also missing								
I. Total phosphorus	•	Röse***	153	A	Before	Yes	13–14	?	5.4 –22.1	15.1	
	•	Clark and Levine	5	R and P A	Both	?	?	?	11 –15.0 10 –12.0	— —	
		Karlström	1	P A	?	Yes	29	Man—"Good health and no known predisposition to disease." "Nearly caries immune." No tarter. Good gums	11.4 –19.2	—	
	•	Roskin	17	R	Before	?	"Children"	Diabetic, caries inactive	8.7 –17.8	12.1	Caries active, non-diabetics. Various diseases
	•	Entin and Geikin	?	R	?	?	?	?	—	14.0	
		Hubbell and Bunting	22	P A,	?	Yes	7–16	Caries inactive children	9.4 –17.0	—	Caries active
			275	P A	(10:00 a.m.)	Yes	?	Caries free school children	—	13.3	
	•	Krasnow, Karshan, and Krejci	13	?	?	?	?	?	8.8 –16.8	—	Tisdall method
			4	?		?	?	?	9.4 –10.9	—	Fiske and Subbarow method
	•	Price	?	?	?	?	?	?	4.55– 6.55	—	
	•	Youngburg	20	? Pooled	?	?	Adult	"Normal"	—	19.32	
	•	Adams and Myers	4	R	After	?	Medical and dental students	"Good teeth and gums"	11 –18	14	"Good, fair and poor teeth and gums." Susceptible to colds

I. Total phosphorus	•	Boyd, Drain, and Stearns	4 12	P A	Before	?	Part of group of 98 = 3-20. Av. = 10 yrs.	"No cavities, no caries" "Inactive caries"	7.2 -12.1 8.8 -17.8	10.2 12.4	"Active caries" Medical history known but not published. Some healthy; some various diseases
		Hubbell	15	P A	Before	Yes	9–15	"Caries free"	11.4 -17.8	14.7	Caries susceptible
	•	Brown and Klotz	18	P A	?	Yes	First year med. students	?	7.9 -21.1	14.2	
		Koehne, Bunting, and Morrell	3	P A	Before	Yes	6–13	"Continued absence of caries"	11.4 -14.1	—	Caries active
			8					Caries inactive	9.5 -15.8		Free from metabolic disturbance
	•	Mathis	1	?	?	?	?	?	—	12.84	
	•	Price	2,600	?	?	?	?	?	4.0 -13.0	10.0	
		White and Bunting	12	R	Before	Yes	9–16	Caries free school children	14.1 -18.1	16.0	Also P A saliva, caries susceptible
	•	Lische	?	?	?	?	?	?	?	14.4	
II. Total acid-soluble phosphorus		Clark and Shell	5	R	2 hours after	Yes	22, 31, 36 42, 49	Good health, see medical history	10.6 -24.0	—	C & S state that in 10 determinations on 5 cases the total acid sol. P = the inorganic P
	•	Smith	9	R	?	?	?	?	10.5 -27.9	—	
	•	Weber	4	?	After	?	20, 35, 41	Clinically healthy	17.3 -29.3	—	
	•	Youngburg	20 Pooled	?	?	?	"Adult"	"Normal"	—	—	Y states only that total acid soluble = inorganic P
	•	Mathis	1	?	?	?	?	?	—	12.80	
III. Inorganic phosphorus	•	Pickerill	1	A	?	Yes	?	?	—	21.0	
	•	Simons	?	?	?	?	?	?	—	19.4	
		Clark and Shell	5	R	After (2 hours)	Yes	22, 31, 36, 42, 49	Good health, see medical history	7.8 -24.0	14.8	
	•	Entin and Geikin	138	R	?	?	Young men, 15–38	Relatively caries immune	9.35-15.15	12.25	Various diseases
			12				?	Caries immune	6.50-15.73	11.59	Clinically healthy

*** In the previous report (7, page 393) the statement concerning Röse's consideration of rate of flow and resting or activated saliva has to be corrected to the effect that he gives detailed records on salivary secretion, calculated for 45 minutes on activated samples.

TABLE II—*Concluded*

1 PHOSPHORUS FRACTION	2 DATA INCOMPLETE	3 AUTHOR	4 NO. OF CASES	5 RESTING OR ACTIVATED SALIVA	6 BEFORE OR AFTER MEAL	7 RATE OF FLOW	8 AGE OF PATIENTS	9 HEALTH OF PATIENTS	10 RESULTS Range	11 RESULTS Av.	12 ADDITIONAL MATERIAL AND REMARKS
									mg. %	mg. %	
		Becks	25	R	Before	Yes	8–71	Caries immune and free from metabolic disturbance	6.2 –11.8	8.9**	Pregnancy
	•	Horton, Marrack, and Price	54	A	?	?	3–12	?	?	?	
	•	Smith	9	R	?	?	?	?	9.2 –25.6	—	
	•	Weber	4	?	After	?	20, 35, 41	Clinically healthy	11.0 –25.0	—	
	•	Karshan, Krasnow, and Krejci	17	P A	Before	?	19–25	Immune to caries	8.8 –15.4	11.3	Caries active
			6				5–15	Arrested caries	8.8 –13.6	11.6	
	•	Axmacher	1	?	?	?	?	?	22.2 –49.2	—	
III. Inorganic phosphorus	•	Forbes	5	R	?	?	?	?	13.5 –19.8	—	Also activated
	•	Forbes and Gurley	2	R	After	?	20–30	Caries inactive	9.5 –17.9	—	Also activated
	•	Krasnow, Karshan, and Krejci	13	?	?	?	?	?	8.7 –17.2	—	Tisdall method
			4						6.3 – 8.3	—	Fiske and Subbarow method
	•	Krasnow	11	R	Before	?	19–21	Medical students, caries immune	10.4 –22.3	16.1	Caries active. Also P A; and 5 p.m. samples
	•	Price	25	?	?	?	?	?	2.5 –11.5	6.0	
	•	Youngburg	92	?	?	?	21–80	"Normal persons"	7.48–32.29	17.50	Caries active
			20				"Adult"		Pooled	18.54	Nephritics
	•	Mathis	6	?	?	?	?	"Healthy persons, free from caries and inflammation of the mucosa"	11.1 –20.4	15.4	
			1						12.26	—	
	•	Price	2,600	?	?	?	?	?	—	5.0	

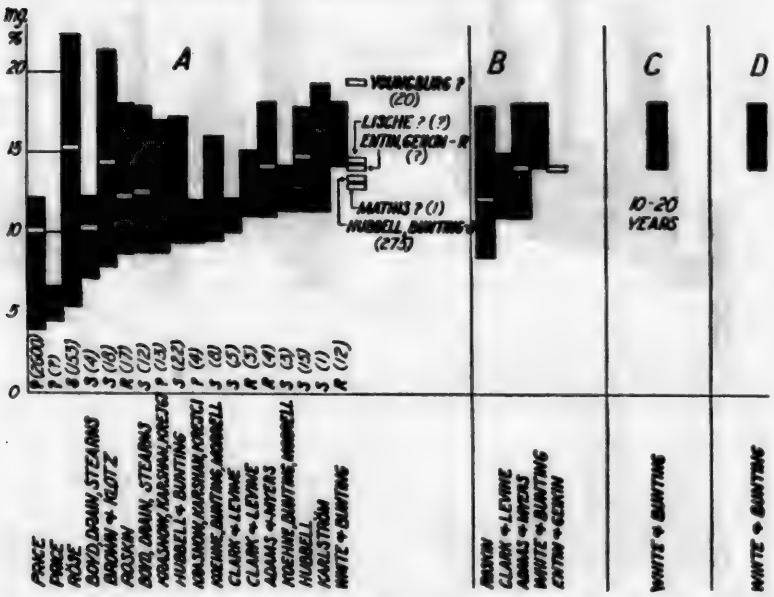
** The original phosphorus values of Becks (5, 6) must be multiplied by 2.

with other fractions in certain experimental considerations and not for comparison with pathological cases nor for comparison with the major portion of the material examined.

Only the analyses of Clark and Shell appear to be acceptable for the establishment of normal standards, because they alone fulfilled

TABLE III

Total phosphorus content of saliva as found by former investigators



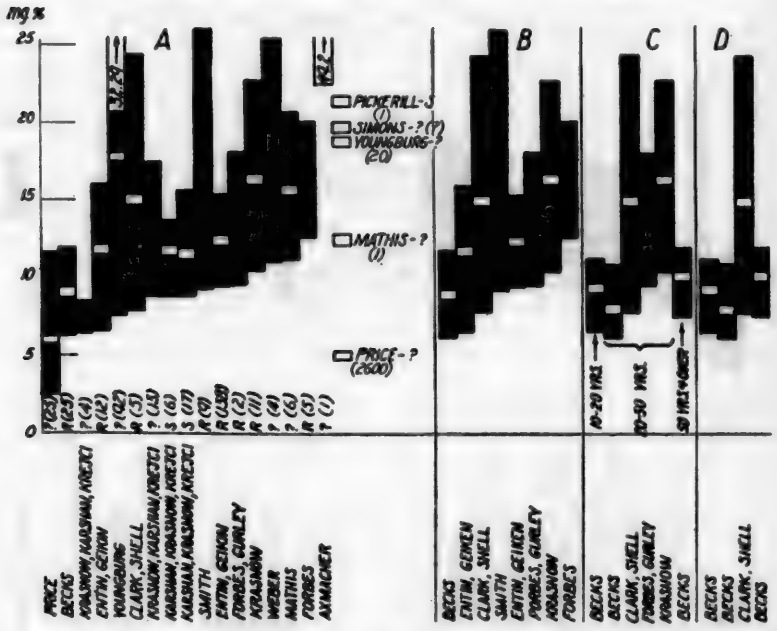
A. Ranges and average values of all authors. B. Ranges and average values of only those authors who used resting saliva. C. Ranges and average values of only those authors under B who paid attention to age of patients. D. Ranges and average values of only those authors under A who reported their material and analytical procedure completely. R: Resting Saliva. S: Stimulated Saliva. ?: Type of Saliva not reported. (no's): Number of cases examined. □: Average. ■: Range.

all technical requirements, which they give in detail. They also took into consideration all factors contained in Table II, columns 5 to 9. The results of Clark and Shell (21) for total acid-soluble phosphorus, ranging from 10.6 to 24.0 milligrams per cent, belong to the age group 22 to 49 years. It is, of course, obvious that the

number of patients examined by them (5) is very small and that it would be necessary to analyze the saliva of a much larger number of patients in order to obtain a reliable average for this phosphorus fraction.

TABLE IV

Inorganic phosphorus content of saliva as found by former investigators



A. Ranges and average values of all authors. B. Ranges and average values of only those authors who used resting saliva. C. Ranges and average values of only those authors under B who paid attention to age of patients. D. Ranges and average values of only those authors under A who reported their material and analytical procedure completely. R: Resting Saliva. S: Stimulated Saliva. ?: Type of Saliva not reported. (no's): Number of cases examined. □: Average. ■: Range.

Group III. Inorganic Phosphorus. The inorganic fraction of salivary phosphorus has been investigated by 18 authors[5] (Tables I, II and IV). Of these, 7 used resting saliva (Table IV, section B)

[5] The publication of Karshan (60) appeared after completion of this manuscript and a discussion of it could not be included.

and 4 separated their patient material into various age groups (Table IV, section C). In Table IV, section D, there are only 2 investigators of section C who give ranges for certain age groups, used resting saliva and also adequately reported their technic and material examined. It should be emphasized here that it is not the authors' intention to doubt the results of all investigators who are not included in section D, but, rather, that it is impossible to evaluate their work and to use it for the establishment of normal standards.

The reasons for omitting in section D all but 2 of the authors becomes quite obvious if one compares the ranges and averages of results in columns 10 and 11 of Table II. Even though the missing data for each author are indicated in Tables I and II by a "?", it seems advisable to comment further on the work of this group in order to avoid any misunderstanding as to other significant features in their work not listed in Table IV.

Pickerill's (90) high average of 21 milligrams per cent in 1 patient can hardly be accepted, because of indefiniteness of his report. Entin and Geikin's (30) publication, which has been widely quoted, contains so many unsubstantiated conclusions that it is necessary to handle each item separately. These authors state that they examined 138 men of a military school and found an arithmetic mean for inorganic phosphorus of 12.25 milligrams per cent. For the biometrical evaluation, however, they used only 121 individuals, and the reader is at a loss to understand how these 121 were selected from the 138. A comparative study of their own results with those of Röse (102) was then made. Röse reported an average of 34.6 milligrams per cent P_2O_5 in 153 cases. Actually, the result of the computation of his values made by Entin and Geikin from P_2O_5 into milligrams per cent phosphorus was incorrect and should have been 15.1 milligrams per cent instead of 16.84 milligrams per cent, which was very close to their own standard average of 12.25 milligrams per cent. They also examined a small number of pathologic cases. Their highly mathematical conclusions, however, are unjustified because the average values for each group show very insignificant deviations from their average norm. The report of these authors is an example of the necessity for not blindly establishing a norm but taking into consideration all of the factors outlined in Table II.

Horton, Marrack and Price (50) reported examination of inorganic phosphorus in 54 children but failed to report their results. Smith (110) examined 9 patients to detect phosphatase activity and its relation to calculus formation but did not consider sufficient factors to make the results of use in this discussion.

The paper of Weber is of special interest because he called attention to the possibility of changes in the amounts of the phosphorus fractions if fresh material is not used for analysis. When saliva was allowed to stand open at room temperature there was increase of inorganic phosphorus at the expense of the organic fraction during the first few hours. Later a reversal of this synthesis could be observed which gave an increased amount of organic phosphorus. Though these changes did not occur in every case, Weber's report has pointed out the necessity of performing the analyses immediately after collection. The criticism expressed at the beginning concerning the frequent lack of information as to which type of saliva was used applies also to Weber (116) who speaks only of the fact that saliva was taken after breakfast without mentioning how many minutes or hours after breakfast. The time elapsed following the meal in the morning can, of course, have an essential influence on the result as shown by Mathis (82); Clark and Shell (21); Forbes (35); Forbes and Gurley (37) and Becks (10) in their studies of daily variations.

The credit for the most comprehensive studies of mixed human saliva, and in particular of the calcium and phosphorus content, goes to the research group of Krasnow, Karshan and Krejci who paid special attention to details of method, selection of material and collection of samples. Karshan, Krasnow and Krejci in their earlier paper of 1931 (59) reported the use of paraffin activated saliva. Later, in 1932 (71), they did not mention what type of saliva was used in their 17 cases, and finally, Krasnow (68) in 1932 reported on 11 cases of resting saliva. How important it is to know exactly what type of saliva is used is demonstrated by the results. Karshan, Krasnow and Krejci report averages of 11.3 and 11.6 milligrams per cent for *paraffin activated* saliva and the average of Krasnow's *resting* saliva is found at 16.1 milligrams per cent, which is about 5 milligrams per cent higher in approximately the same age group.

Axmacher (7) analyzed the saliva of only 1 person and reports

much higher values than most of the other authors of this group (22.2 to 49.2 milligrams per cent). The papers of Forbes (35) and Forbes and Gurley (37), which deal with the effect of the neutralizing power of *saliva*, contain data on the inorganic phosphorus content. The values of Forbes on 5 patients cannot be used because data as to age and health of patients are missing. In these reports no attention has been paid to rate of flow. Price's extensive publications (94, 95, 96, 97, 98, 99, 100) on salivary composition in relation to the denture could not be evaluated by the authors because data on the technical procedure, as indicated in Tables I and II, have not been published as yet and most essential data as to the material used are missing. In view of the fact that a large number of patients, 2600, (101) were examined by him, the authors feel that it would be a great loss to the profession if these data remain unpublished because without them the values cannot be used for comparative study.

The work of Youngburg (118), the originator of a colorimetric method, requires special attention. He reports analysis of inorganic phosphorus for 92 "*normal persons*" between the ages of 21 and 80 years with a range of 7.48 to 19.99 milligrams per cent. Youngburg's Table I, however, indicates a range from 7.48 to 32.29 milligrams per cent phosphorus (average 17.50 milligrams per cent) for this group. Youngburg furthermore quotes 12 cases of Entin and Geikin, for which an average "normal" value of 11.59 milligrams per cent inorganic phosphorus was found, not mentioning their main group of 138 patients for which they established a range from 7.41 to 24.23 milligrams per cent and a *normal average* of 12.252 ± 2.901 (9.35 to 15.15 milligrams per cent). Youngburg does not mention the rate of flow. If we compare the average results in the various age groups of Youngburg's series, it can be noticed that the inorganic phosphorus content increases with advancing age; this is in agreement with a former report of Becks (10). However, the average value of 17.50 milligrams per cent for the ages from 21 to 80 years is a misleading figure if applied as a norm to lower and higher age groups. The same criticism applies to Becks' average.

Mathis (82) examined altogether 7 individuals, for whom he did not give age, rate of flow, nor state whether resting or activated saliva was used. Peculiarly enough he leaves out the description of the health

of these patients in his original publication but it is given in his abstract (83). It is difficult to believe that any serious biologist would try to establish normal standards on the examination of 6 individuals, even though "numerous" analyses were made of mixed saliva from these 6 persons. Since some of the most essential data are missing from Mathis' work, the range of his results cannot be accepted.

The survey of this Group 3 reveals, therefore, that in the final analysis only 2 reports can be accepted for the establishment of normal standard values for inorganic phosphorus fraction, those of Clark and Shell; and Becks. Since the range and average values differ considerably, additional data will be needed, which must be obtained under controlled conditions.

SUMMARY AND CONCLUSIONS

This study of the approach of former investigators brought out the fact that our knowledge of the phosphorus content of saliva is less than meager. Very frequently statements were found of "high and low" phosphorus values. However, can it be possible to speak of high and low values if no one has succeeded as yet in establishing norms?

The main difficulties and deficiencies in former calcium studies were in the analytical procedure, which influenced the results to a large extent. In the case of phosphorus, however, the differentiation into the various fractions represented an additional difficulty.

For this reason not only considerable confusion exists in the literature as to the terminology but also as to the results. If we should accept the work of all authors blindly we would find the following ranges (lowest and highest values of all authors of each group) as taken from Table II:

Total Phosphorus	=	4.0 to 22.1 mg. per cent
Total Acid-Soluble	=	10.5 to 29.3 mg. per cent
Inorganic Phosphorus	=	2.5 to 49.2 mg. per cent

These ranges might be considered perfectly possible and typical for the norms, however, we feel, for reasons discussed, that in the establishment of standard norms the work of only a few authors can be considered.

For the survey of the work on total phosphorus content of saliva, 18 reports were available. The list of those acceptable for normal standards finally narrowed down to the work of White and Bunting, who reported a range of 14.1 to 18.1 milligrams per cent total salivary phosphorus in 12 individuals of 10 to 20 years of age.

For the total acid-soluble fraction, for which 5 papers were available, only Clark and Shell have reported reliable data, with a range of 10.6 to 24.0 milligrams per cent in 5 individuals between the ages of 22 and 49 years. Eighteen authors determined the inorganic phosphorus fraction of saliva, of which only Clark and Shell; and Becks presented complete records. The former reported a range of 7.8 to 24.0 milligrams per cent (average 14.8 milligrams per cent) for 5 individuals between 22 and 49 years of age, while Becks found a lower range for 25 individuals (18 to 71 years), from 6.2 to 11.8 milligrams per cent (average 8.9 milligrams per cent). The analytical results for various phosphorus fractions found in pathologic cases, caries included, were omitted and will be given further consideration in a subsequent publication.

We are in full agreement with the suggestion of Krasnow and collaborators as to the preparation of the patient previous to the examination of their saliva. It is necessary to have a thorough description of health and metabolic status of the patient, data as to whether resting or activated saliva has been used, with special reference to rate of flow. For the interpretation of results additional information on the age, sex, nationality, and detailed dental and nutritional history must be given.

It is quite obvious that this lack of adequate data on the salivary phosphorus composition is a definite handicap in the understanding of some of our most important problems: whether salivary phosphorus has any relationship to dental caries, paradentosis or calculus formation.

REFERENCES TO LITERATURE

References for this paper and the two succeeding papers have been combined and appear at the end of the last paper (p. 247).

HUMAN SALIVA

IV. Critical Discussion of Former Studies of the Distribution of Salivary Phosphorus Fractions[1]

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INTRODUCTION

In the course of the study of salivary phosphorus (12) it became evident that for diverse reasons the results of former investigators were not comparable or acceptable. Factors which can definitely affect the results, i.e., age and health of the individuals examined, rate of secretion, and certain technical phases of analytical procedure, were not reported or considered in the interpretation and evaluation. One of the outstanding difficulties in the interpretation of published results was the fact that most authors did not differentiate between the various phosphorus fractions present in saliva. For this reason and because in many instances the fundamental principles of phosphorus analysis apparently were not generally understood, the percentage distribution of certain of these fractions is still not known. Clark and Shell (21), for instance, stated that the total acid-soluble fraction of mixed human resting saliva equaled the inorganic phosphorus fraction. In a later publication this work was cited by Clark and Levine (20) as evidence that the total phosphorus of saliva is entirely in the inorganic form. Kesel (61), in a review of the caries problem, used this statement as an argument against the conclusions of Price. He states, "Clark and Shell in reporting a carefully conducted study of the influence of diet on the inorganic constituents of human saliva make the following statement: '—after about a third of the determinations had been completed, it was found that the

[1] Conducted under grants made by the California State Dental Association, California Academy of Periodontology, Research Board of the University of California and the Good Teeth Council for Children Inc.

phosphorus of the saliva was present entirely in the form of inorganic phosphate.' If their finding is correct, Dr. Price's speculation in regard to activators releasing the bond of phosphorus with organic compounds is not tenable." This illustrates the extent of the misunderstandings in the terminology of the phosphorus fractions and the influence of these misapprehensions upon the interpretation of findings. Innumerable other incidents could be quoted in which authors used incorrect terms for the phosphorus fractions. Oftentimes these irregularities in terminology can be easily discovered by an examination of the described methods (Table III), however, in some instances neither the description of the fraction analyzed nor the report of the method used are adequate to allow the reader to determine the fraction under scrutiny.

In view of the complexity and contradictory nature of the reports for qualitative and quantitative results of the total phosphorus content and other fractions of saliva, it was determined necessary to compare the various methods used by former authors before any consideration was given to their results.

FUNDAMENTAL PRINCIPLES OF PHOSPHORUS ANALYSIS

According to present knowledge it appears that salivary phosphorus occurs in more than one form. The known fractions may be classified as follows: *total* phosphorus, *total acid-soluble* phosphorus, *inorganic* phosphorus, *total organic* phosphorus, *acid-insoluble organic* phosphorus, *acid-soluble organic* phosphorus and phospholipids. In most modern methods the type of preliminary treatment given the organic matter determines which of these fractions is analyzed.

For the determination of the total phosphorus content, either of the standard wet or dry ashing procedures may be used on the whole saliva; but the total acid soluble phosphorus requires the preliminary removal of protein by precipitation with trichloracetic acid, followed by the ashing procedure. It is apparently the opinion of some that the entire total phosphorus is total acid-soluble; if this were the case the trichloracetic treatment would be superfluous. The inorganic fraction is determined by direct analysis of the trichloracetic filtrate without ashing. The organic fractions, which have found comparatively little consideration in analytical work, are determined

indirectly by calculation. The fraction most frequently mentioned is the *total organic phosphorus*, which is composed of the organic *acid-insoluble* and the organic *acid-soluble* parts. The organic *acid-insoluble* fraction includes the phosphorus derived from mucin, nucleoprotein of epithelial cells, and phospholipids; and the *organic acid-soluble* fraction includes the phosphorus from pyrosphosphate, creatine phosphate, hexosemonophosphoric acid, and diphosphoglyceric acid. The values are calculated as follows:

total organic phosphorus = total phosphorus − inorganic phosphorus

organic acid-insoluble phosphorus =
total phosphorus − total acid-soluble phosphorus

organic acid-soluble phosphorus =
total acid-soluble phosphorus − inorganic phosphorus

These simple equations explain the difference between the fractions and how they can be estimated. With these few fundamentals in mind it is not very difficult to analyze the work of former investigators and classify their reports. It was found that in a number of instances an entirely different fraction than the one reported had been determined, which, of course, is most misleading.

After the isolation of the fraction the measurement of the phosphorus present can be accomplished in 2 different ways: first, by precipitation of the phosphorus as ammonium phosphate, ammonium phosphomolybdate, etc., followed by a gravimetric, volumetric, or gasometric determination (Table I); second, without preliminary precipitation by direct analysis of the saliva or, more commonly, of trichloracetic filtrate (Table II). Colorimetric methods of the later type, which depend upon the development of a blue color by the reduction of phosphomolybdate, are most frequently used, though in some biological materials this color development can be influenced by certain factors to such an extent that its application is questionable. The two groups of methods, as outlined by Peters and Van Slyke (89), are summarized in Tables I and II. The names of the authors who applied a given method to saliva have been listed in the last column.

Of the procedures based on primary precipitation of the phosphorus, the gravimetric methods were among the earliest. Modern refinements have made them suitable for microtechniques and allow the

TABLE I

Methods of phosphorous analysis of biological material requiring preliminary isolation of phosphorus

TYPE	PRECIPITATED AS	WEIGHED AS	MILLIGRAMS P	APPLIED TO	MODIFICATIONS USED AT PRESENT	APPLIED TO SALIVA BY
A. Gravimetric	1. Magnesium ammonium phosphate	$Mg_2P_2O_7$	10–100	Urine, feces		Karlström (57, 58)
		$MgNH_4PO_4 \cdot 6H_2O$			Jones (56)	
	2. Ammonium phosphomolybdate	$(MoO_3)_{12}PO_4 \cdot (NH_4)_3(HNO_3)_3 \cdot H_2O$			Lorenz (79, 80) Lieb (77) Neubauer (86) Kuhn (73)	Mathis (82)
	3. Strychnine phosphomolybdate	Strychnine phosphomolybdate	0.1–4	Blood, urine and feces	Embden (27)	
		TYPE OF TITRATION				
B. Volumetric	1. Magnesium ammonium phosphate	Acidimetric	2–7	Urine	Fiske (33)	
	2. Ammonium phosphomolybdate	Alkalimetric	5–20	Feces	Neumann (87) Shohl and Brown (108) Samson (105)	Adamson (3) Entin and Geikin (30)
	3. Strychnine phosphomolybdate			Blood, urine and feces	Myrbäck (85)	
	4. Ammonium phosphomolybdate	Permanganate			Javillier and Djelatides (55)	
		REDUCING AGENT				
C. Colorimetric	1. Ammonium phosphomolybdate	Phenyl hydrazine			Taylor and Miller (111)	
	2. Strychnine phosphomolybdate	Ferrocyanide	0.02–0.1	Blood, urine and feces	Tisdall (112)	Krasnow, Karshan, Krejci (71) Karshan, Krasnow, Krejci (59) Krasnow (68)
		GAS LIBERATED				
D. Gasometric	1. Strychnine phosphomolybdate	CO_2	0.005–0.2	Body fluids	Kirk (62)	

determination of very small amounts—as small as 0.1 mg. Thus, the gravimetric methods are applicable, under certain conditions, to salivary work. The classical method is to weigh the magnesium ammonium phosphate precipitate, either dry ashed to $Mg_2P_2O_7$ or dried to constant weight as $MgNH_4PO_4 \cdot 6\ H_2O$. This can be applied when amounts of phosphorus from 10 to 100 mgm. are available. An improvement, introduced by Lorenz (79), was made by precipitating the phosphorus as ammonium phosphomolybdate. This compound, $(MoO_3)_{12}\ PO_4(NH_4)_3\ (HNO_3)_2 \cdot H_2O$, has a higher molecular weight (2019), thus giving a precipitate of larger mass. In some methods the molybdate precipitation is used only to separate the phosphorus from substances which might interfere with its isolation as, for example, magnesium ammonium phosphate. It was later found by Pouget and Chouchak (92) that phosphorus could be precipitated as strychnine phosphomolybdate. Embden (27) defined conditions for its use in gravimetric methods, where it is particularly suitable because of the very large molecular weight. The weight of strychnine phosphomolybdate containing 1 mg. of phosphorus is 89.3 mg., whereas that of ammonium phosphomolybdate is 69 mg. The strychnine precipitate is more insoluble, precipitates more rapidly in large particles and can be easily dried to constant weight in an oven at 100 to 110°C. Fetter (32) and Myrbäck (85) have adapted this procedure to analysis of blood filtrates from plasma in amounts as small as 2.5 ccm.

The *volumetric* methods in this group (B Table I) are of 3 types. The *first* by Fiske (27) depends upon acidimetric titration of the magnesium ammonium phosphate precipitate. Hydrochloric acid is used and each molecule of phosphate neutralizes 2 equivalents of acid. This method is somewhat tedious but can be used for 2 to 7 mg. of phosphorus. In the *second* volumetric method the ammonium phosphomolybdate precipitate is titrated alkalimetrically. With this precipitate 1 molecule of phosphoric acid neutralizes 28 equivalents of alkali. This method has been used on samples as small as 0.1 mg. phosphorus. Modifications have been published by Bang (8); Hammarsten (40); Kuhn (73); Pemberton (88); Shohl and Brown (108); Neumann (87); and Samson (105). Alkalimetric titration has been applied to the strychnine phosphomolybdate precipitate

by Myrbäck (85). This technic, however, has not given consistent results in the hands of Peters and Van Slyke (89). The *third* volumetric method is that of permanganate titration of the molybdenum in the ammonium phosphomolybdate precipitate according to the technic of Javillier and Djelatides (55). This is a rather tedious method but can be applied to amounts of phosphorus as small as 0.5 mg. with an error of usually less than 1 per cent.

These precipitates may also be determined *colorimetrically* (C Table I) where quantities of phosphorus of less than 0.1 mg. are encountered, as in blood and saliva. Taylor and Miller (111) redissolve the ammonium phosphomolybdate precipitate and reduce the molybdate ion by phenylhydrazine. Tisdall (112) redissolved the strychnine phosphomolybdate and reduced the molybdate ion with ferrocyanide. In the hands of Peters and Van Slyke (89) this method has given results within an experimental error of ± 2 to 3 per cent.

The strychnine phosphomolybdate precipitate has been utilized by Kirk (62) in a gasometric method (D Table I) performed on very small amounts of phosphorus in the Van Slyke gas apparatus.

Methods of the second main group (Table II) do not require preliminary precipitation of phosphate. This group includes several colorimetric methods but only 1 standard volumetric procedure (A Table II), originally described by Pincus (91). In this volumetric method the phosphate is titrated with uranium acetate or nitrate. The end point is detected by the use of the following indicators: ferrocyanide, cochineal (Malot (81)) and sodium salicylate (Du Parc and Rogovine (25)). This method is usually applied when from 10 to 50 mg. phosphorus are contained in the sample, as in urine and feces.

By far the most popular micro methods of this group (B Table II) are those depending upon the colorimetric reading of the blue color produced by the reduction of phosphomolybdate. These methods can be applied on amounts of phosphorus varying from 0.01 to 0.1 mg. Although the experimental error will fluctuate in some of these as much as ± 5 per cent, they offer very rapid and simple procedures less subject to the errors incident to complicated and tedious methods. Bell and Doisy (13) introduced a very satisfactory technic that depends on the reduction of phosphomolybdate by hydroquinone with

sulphite in the presence of sodium carbonate. Briggs (18) observed that in acid solution hydroquinone developed a stronger color, while Benedict and Theis (14) produced an even stronger color development by heating the final solution in boiling water. In another very

TABLE II

Methods of phosphorus analysis of biological materials by direct determination (without preliminary precipitation of phosphorus)

TYPE	TITRATED WITH		MILLIGRAMS P	APPLIED TO	MODIFICATIONS USED AT PRESENT	APPLIED TO SALIVA BY
A. Volumetric	Uranium acetate		10–50	Urine and feces	Pincus (91) Malot (81)	Pickerill (90) Simons (109)
B. Colorimetric	REDUCING AGENT					
	1. Hydroquinone	a. Sodium carbonate	0.02–0.1	Blood	Bell-Doisy (13)	Clark and Shell (21) Becks (9, 10)
		b. Acid solution	0.02–0.1	"	Briggs (18)	Hawkins (46, 47, 48) Adamson (2)
		c. Plus heat	0.02–0.1	Blood except cell inorganic P	Benedict-Theis (14)	Clark and Levine (20) Roskin (103) Boyd, Drain and Sterns (17)
	2. Stannous chloride		0.01–0.04	Blood	Kuttner and Cohen (74) Kuttner and Lichtenstein (75) Youngburg (118) Bodansky (16)	Price (96) Youngburg (118) Brown and Klots (19)
	3. Aminonaphthol-sulfonic acid		0.02–0.1	"	Fiske and Subbarow (34)	*
	4. Monomethyl-p-amino-phenol		0.02–0.1	"	Leiboff (76)	

* Eddy, Heft, Rosenstock and Ralston (26); Hubbell and Bunting (52); Krasnow, Karshan and Krejci (71); Adams and Myers (1); Hubbell (51); Koehne, Bunting and Morell (67); White and Bunting (117); Smith (110); Weber (116); Becks (9, 10); Axmacher (7); Forbes (35); Forbes and Gurley (37).

rapid technic, originally developed by Denigés (23), the powerful agent stannous chloride was introduced for the reduction of phosphomolybdate. This technic has been refined recently and applied to biological material by several authors, including Kuttner and Cohen

(74); Kuttner and Lichtenstein (75); Youngburg (118); Atkins and Wilson (6); and A. Bodansky (16). Possibly the most popular of these colorimetric methods was introduced by Fiske and Subbarow (34) in 1925. These authors used as the reducing agent, aminonaphtholsulfonic acid, which must be prepared fresh about every 2 weeks. In spite of this inconvenience the method offers a very simple and speedy procedure and reduces the number of solutions required for analysis. Leiboff (76) has used monomethyl-p-aminophenol for the same purpose, stating that it is a very stable reagent. As yet few investigators have adopted this method and little information is available concerning its use and applications. All these colorimetric methods give perfectly satisfactory results, but care must be exercised in their choice and application to the specific material to be examined.

The methods used by the different investigators of human saliva are indicated in Tables I and II. Few used preliminary precipitation, the method described in Table I Only Karlström (57, 58) and Mathis (82) used a gravimetric method with preliminary precipitation of the phosphorus. Entin and Geikin (30) used a volumetric and Krasnow, Karshan and Krejci (71) a colorimetric procedure. It might be added that the last colorimetric procedure (112) is exceptionally accurate and does not require as large amounts of phosphorus as do the methods used by Karlström and by Entin and Geikin. It is also faster than the microgravimetric method used by Mathis.

The determination of phosphorus directly on the trichloracetic filtrate without the preliminary precipitation of the phosphorus was employed by a number of investigators (Table II). The 1 volumetric method in this group was used by only Pickerill (90) and Simons (109). It is an older method and does not seem suitable for salivary analysis because of the large amount required for a single determination.

The colorimetric method of Bell-Doisy (13), and its modifications, was used by six authors: Clark and Shell (21); Becks (9, 10); Hawkins (46); Clark and Levine (20); Roskin (103) and Boyd, Drain and Stearns (17). These methods are accepted everywhere in biochemical work as the oldest dependable colorimetric methods. The stannous chloride procedure was used by Price (96); Youngburg (118); and Brown and Klotz (19). It is more sensitive and requires smaller

amounts of saliva but must be performed with great care. The Fiske and Subbarow (34) method was employed by 13 investigators, which is almost half of all the modern investigators of saliva. It is accepted in all biochemical fields where a micro method is needed.

Summarizing, it can be stated that, with the exception of the uranium acetate procedure (Table II) used by Pickerill and Simons, all methods used by former investigators of saliva are acceptable for salivary work. The most popular are the methods of Tisdall; Briggs, Bell and Doisy; Fiske and Subbarow; and the stannous chloride procedure. These appear to be preferable to other known procedures and a comparison of the results obtained with them in an experimental study will be discussed in the next paper of this series.

DISTRIBUTION OF SALIVARY PHOSPHORUS FRACTIONS

In establishing normal standards for the various phosphorus fractions in mgm. per cent (12) it was found necessary to eliminate for the total phosphorus all but 1 (White and Bunting) of 18 reports; for total acid soluble all but 1 (Clark and Shell) of 5 reports; and for inorganic phosphorus fractions all but 2 (Clark and Shell; and Becks) of 18 reports. Most of these reports were not accepted because of inadequate description of material, age, sex, health of individual, rate of secretion, methods of analysis, activation or non-activation of saliva, etc. These authors used the technical analytical methods of Fiske and Subbarow (White and Bunting, and Becks) and Bell and Doisy (Clark and Shell, and Becks), which were mentioned before as being preferable. However, it may be emphasized again that they apply only to solutions in which the actual phosphorus component to be analyzed has been isolated previously. Ignorance of this fact has led many investigators to publish results which do not correspond to the fraction specified, and, in some cases, the designation indicated that the investigator was not aware of the fact that there was more than one fraction of salivary phosphorus. Very few authors could be found who evinced interest in more than one phosphorus fraction in the same saliva sample and a calculation of their percentage distribution (Table III). The authors in Table III are taken from Tables I and II of a former publication (12), where their technical approach and results in mg. per cent were reported. In several

TABLE III

Percentages of various salivary phosphorus fractions as found or calculated by former investigators

GROUP	AUTHOR	YEAR	PRELIMINARY CENTRIFUGATION	RESTING OR ACTIVATED SALIVA	NO. OF CASES	(1) TOTAL P.	(2) TOTAL ACID-SOLUBLE P	(3) INORG. P.	(4) TOTAL ORGANIC	(5) ORGANIC ACID-INSOLUBLE P	(6) ORGANIC ACID SOLUBLE P	(7) PHOSPHO-LIPIDS
						Method of preliminary treatment			Calculated by difference			
						Ashed (wet or dry)	Trichloracetic filtrate		(1) − (3) = (5) + (6)	(1) − (2)	(2) − (3)	Alcohol-ether extraction
							Wet ashed	Direct				
						per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I	Clark and Shell	1927	No	R	5	—	X[1]	100 of (2)	—	—	0	—
	Entin and Gelkin	1928	No	R	12	Calculated	—	83	—	17	—	—
	Adamson	1929	?	?	1	—	X	83 of (2)	—	—	14.3–20 Av. 17 of (2)	—
	Smith	1930	No	R	9	—	X	91 of (2)	—	—	4–17 of (2) Av. 9	—
	Weber	1930	No	?	4	—	X	41–98 of (2)	—	—	2–59 of (2)	(8.5–14.0 mg.%)
II	Axmacher	1932	No	?	1	100	—	87	13	—	—	—
	Krasnow, Karshan and Krejci	1932	No	?	4[2]	100	—	70	30	—	—	—
					13[3]	100	—	100	0	—	—	—
	Youngburg	1932	No	?	20[4]	100	96	96	4	4	0	0
	Krasnow	1934	?	?	40	—	—	—	—	—	—	(0.1–0.4 mg.%)
	Mathis	1934	No	?	1	100	100	95.5	4.5	0	4.5	0
	Krasnow and Rosen	1936	?	?	80	—	—	—	—	—	—	(0.05–1.79 mg.%)
	Price	1936	?	?	2,600	100	—	50	50	—	—	—

[1] X: Determination made for total acid-soluble phosphorus but not for total phosphorus.
[2] Fiske and Subbarow method.
[3] Tisdall method.
[4] Includes the P derived from: mucin, nucleoprotein of epithelial cells and phospholipids.
[5] Includes the P derived from: pyrophosphate, creatine phosphate, hexosemonophosphoric acid, dyphosphoglyceric acid.
[6] Pooled.

instances only 2 or 3 fractions were determined and the others calculated therefrom. Most of these reports have appeared since 1927.

In comparing the percentages found or calculated for Group I, the total acid soluble phosphorus was taken as 100 per cent because

the authors of this group did not examine the total phosphorus. In Group II all calculations are based on the total phosphorus taken as 100 per cent. The columns on the left of Table III are arranged in chronological order; they give the names of investigators, the year of publication and the number of cases examined for more than 1 fraction. Most of these comparative examinations of the phosphorus fractions were made on groups of from 1 to 20 cases, with the exception of Price who reported on 2,600 (101).

Four authors of Group I (Clark and Shell (21); Adamson (3); Smith (110); and Weber (116) reported the examination of *total acid-soluble phosphorus* (Column 2 Table III) They gave their values in mg. per cent without analyzing the total phosphorus content, thus making it impossible to estimate the percentages on the basis of total phosphorus content, as was done for Group II. For this reason all calculations of the remaining fractions in Group I were based on these total acid-soluble phosphorus values, which were arbitrarily taken as 100 per cent.

Under Column 1 of Table III it can be seen that only the authors of Group II (Axmacher (7); Krasnow, Karshan and Krejci (71); Youngburg (118); Mathis (82); and Price (101) made actual determinations of the *total salivary phosphorus*, which was accomplished by the preliminary use of a standard ashing method (either wet or dry). This process converts all of the phosphorus in the sample to the inorganic form, in which it can be easily handled by the reagents for quantitative estimation of phosphorus. In addition to these 5 authors Entin and Geikin (30) of Group I report findings for total phosphorus, but they analyzed only the inorganic and the organic acid-insoluble phosphorus and then called the sum of these the total phosphorus, which does not include the organic acid-soluble phosphorus. Hence their value for total phosphorus cannot be accepted.[2]

[2] Scoz (106) reports values for salivary "total phosphorus" ranging from 18.1 to 21.0 milligrams per 100 grams in 3 cases. Although Scoz gives values also for orthophosphoric acid, pyrophosphoric acid and organic phosphorus, the percentages of these fractions have not been calculated or entered in Table III for several reasons. Scoz does not describe his method of phosphorus analysis or his methods of preliminary treatment for the determination of the fractions. The units of phosphorus content are given in "mg. per gm." of saliva, which is not the generally accepted standard of measurement. For these reasons it is not possible to compare his values with those of the other authors of Table III.

The *inorganic fraction* was analyzed by all authors listed in Table III, Column 3. It was determined by the direct analysis (without ashing) of the deproteinized filtrate. Since 5 of these authors also analyzed the total phosphorus content, the percentage of inorganic phosphorus can be given as follows: Krasnow, Karshan and Krejci (71) by the Tisdall (112) method—100 per cent; Youngburg (118)—96 per cent; Mathis (82)—95.5 per cent; Axmacher (7)—87 per cent; and Price (101)—50 per cent. Entin and Geikin's (30) value of 83 per cent is questionable because of the unacceptable total phosphorus calculation. The remaining 4 authors determined only the *total acid-soluble* content, from which the inorganic percentage was calculated by us. The results range as follows: Clark and Shell (21)—100 per cent; Smith (110)—91 per cent; Adamson (3)—83 per cent; Weber (116)—41 to 98 per cent.

The organic fractions (total organic, organic acid-insoluble, and organic acid-soluble) can be calculated directly from the values of columns 1, 2 and 3 of Table III. The formulas for these calculations are given on page 219 under "Fundamental Principles." Entin and Geikin (30) were the only authors to determine an organic fraction by direct analysis as noted below.

It is not surprising that the *total organic* phosphorus content of saliva cannot be calculated from the data of several authors because the total phosphorus was not determined. Krasnow, Karshan and Krejci (71) report a very peculiar discrepancy between the Fiske and Subbarow and the Tisdall methods. While the latter gave 0 per cent, they found with the former as high as 30 per cent. Krasnow (70) explains this on the basis of poor color development found in the case of the Fiske and Subbarow method. They offer no explanation for the complete absence of organic matter by the Tisdall procedure. Youngburg (118) found 4 per cent; Mathis (82) 4.5 per cent; Axmacher (7) 13 per cent; and Price (101) even 50 per cent. In other words, there is a range of 0 to 50 per cent in the results of the total organic portion; it is difficult to reconcile this extreme variation on any known basis. It must be necessarily concluded that not all of the fundamentals of analytical procedure were consistently followed or there were errors introduced by miscalculation.

For *organic acid-insoluble*, Mathis (82) found 0 per cent; Youngburg (118) 4 per cent; and Entin and Geikin (30) 17 per cent of the sum of organic acid-insoluble phosphorus (determined directly on the ash of the trichloracetic precipitate) and inorganic phosphorus. The results of *organic acid-soluble* calculations varied from 0 per cent of Youngburg and Clark and Shell (21) to the 4.5 per cent of Mathis. Since Smith, Adamson and Weber did not determine the total phosphorus content of saliva, their values for organic acid-soluble phosphorus are expressed as percentage of total acid-soluble phosphorus, which amount to 9 per cent for Smith (110), 17 per cent for Adamson (3) and 2 to 59 per cent for Weber (116). Adamson and Smith designated the organic acid-soluble part simply as the organic phosphorus. The values of these 2 authors are, therefore, entered in Column 6 and not Column 4 of Table III. There are several possible causes to which this variation of 0 to 59 per cent may be attributed; such as, the use of preliminary centrifugation, deficiency in analytical procedure, number of cases, variation in amount of epithelial cells, debris content, etc. It was not possible to determine the rôle of these factors from the published data. It seems sufficient to say that both our knowledge and the collection of analytical material of the total organic phosphorus and its 2 subdivisions are inadequate.

Five authors estimated the *phospholipids* (Column 7 of Table III), which are a fraction of the acid-insoluble organic phosphorus. They are determined directly on the alcohol-ether extract of the whole saliva. This extract is wet ashed and the determination continued as for total phosphorus. Youngburg (118) and Mathis (82) found 0 per cent. Other authors expressed their findings in mg. per cent. Krasnow (69) reported 0.1 to 0.4 mg. per cent. Krasnow and Rosen (72) 0.05 to 1.79 mg. per cent and Weber (116) found the extremely high figures of 8.5 to 14.0 mg. per cent. It is impossible to account for this variation on the basis of their reports and indicates the need for further investigation.

The discrepancies of results within the various fractions suggest that in the determinations and calculations several factors can influence the establishment of percentages; outstanding is the technical approach. The same deficiencies in technique were found in the

consideration of the results in mg. per cent of calcium analyses (11) where none of the reports, excepting that of Clark and Shell (21), could be accepted for the establishment of normal standards.

One must suspect a difference, especially in the organic fractions, between resting and activated saliva, between centrifuged and uncentrifuged saliva and between the saliva of adults and children. If these factors of possible influence are disregarded, differences in the percentage distribution must be expected. It has been our concept that centrifugation influences the results to some extent, and an attempt was made to examine its effect. For this purpose centrifuged and uncentrifuged samples of 5 different cases were examined for the total phosphorus (both by wet and dry ashing), total acid-soluble and inorganic fractions. The percentages were calculated in 2 ways

TABLE IV

Average percentages of various phosphorus fractions for five individual cases

	TOTAL P.	TOTAL ACID-SOL. P.	INOR. P.	ORGANIC P.		
				Total	Acid-insol.	Acid-sol.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Centrifuged	100	101.0	94.9	5.1	−1.0	6.1
Uncentrifuged	100	98.0	91.0	9.0	2.0	7.0

(Table IV). First, the total phosphorus content of the centrifuged samples was taken as 100 per cent, and, second, the total phosphorus of the uncentrifuged saliva was taken as 100 per cent. It can be readily noted that there is very little percentage difference in Columns 1 and 2, and in Column 3 the difference is about 4 per cent. In calculating the organic phosphorus fraction of centrifuged and uncentrifuged saliva the only distinct difference appears in Column 4, with the total organic phosphorus of uncentrifuged saliva definitely higher than in centrifuged saliva. The percentage differences in columns 5 and 6 are small.

On the basis of these 5 samples it can be concluded that as far as percentage calculations of the individual phosphorus fractions are concerned, there is little difference between centrifuged and uncentrifuged samples of the same saliva. However, the fact should be

stressed that even though the centrifugation process does not seem to affect the percentages, it lowers the actual results in mg. per cent very decidedly. (To be reported separately.) Since the centrifuged samples are freed from contaminating material, which frequently leads to fluctuations in the results, the centrifugation process was adopted in this laboratory for phosphorus analysis; so all future calculations of percentages are based on the results obtained for centrifuged samples. It seems, therefore, that the average distributions of the various phosphorus fractions are as follows: total phosphorus, 100 per cent; total acid-soluble, 100 per cent; inorganic phosphorus, 95 per cent; total organic phosphorus, 5 per cent; organic acid-insoluble, 0 per cent; organic acid-soluble, 5 per cent.

If the analytical figures of Table IV are compared with those of the authors in Group II, Table III, who based the calculations of their values on the total phosphorus taken as 100 per cent, it can be noted that only the values of Axmacher (7) and Mathis (82) approximate them. It is surprising to note that Price (101) found as much as 50 per cent total organic phosphorus. Since he did not determine the total acid-soluble phosphorus one wonders what the percentages for acid-soluble and acid-insoluble organic phosphorus fraction would be if calculated. Youngburg's (118) pooled sample is hardly comparable. Krasnow, Karshan and Krejci (71) found in 4 samples 30 per cent total organic phosphorus and in an additional 13 cases 0 per cent. Since they did not accept their own values found by the Fiske and Subbarow method because of interference with color development in the inorganic fraction, the 0 per cent of total organic phosphorus for thirteen cases seems to be their concept of the real distribution, which is in variance with the findings of all other authors as well as those in Table IV.

It is questionable whether the various phosphorus fractions are always present in the same proportion. Even though attempts have been made by previous investigators (Table III) to establish percentages of salivary phosphorus fractions, only the values of Axmacher (7) and Mathis (82) seem to represent the true condition in saliva, but they cannot be accepted for reasons explained previously (12).

For the sake of completeness mention must be made of the enzyme salivary phosphatase. In recent years this enzyme has been the subject of several studies because of its suspected rôle in the availability of inorganic phosphorus for chemical processes in the oral cavity. Demuth (22) in 1925 reported the presence of small amounts of an hexose-diphosphate hydrolyzing enzyme in saliva. In 1929 Adamson (2, 3) discussed the action of enzymes in calculus formation. He concluded that: human gum tissue contains an active enzyme which will hydrolyze glycerophosphoric ester; saliva and blood serum contain phosphoric esters capable of being hydrolyzed by the enzyme; the organic phosphate of saliva (the phosphoric ester) is probably of the hexosephosphoric group; human gum tissue does not contain pyrophosphatase. From his observations Adamson concludes further that the phosphatase of gingival tissue can be released by trauma and then by its action on the organic phosphate of saliva release inorganic phosphate. The latter is then, under favorable conditions, precipitated as salivary calculus. Adamson did not refer to the previous work of Demuth. Smith followed Adamson in 1930 (110) and found no relationship between the amount of calculus deposited in the mouth and the organic phosphate of resting saliva. Smith did find phosphatase enzyme present in the desquamated epithelial cells of saliva. The optimum pH for the activity of the enzyme was found to be 5.5. By the use of gingival epithelium Smith was able to deposit calcium phosphate in vitro but was unable to show a similar deposit in gingival tissue in the mouth. Of the previous work Smith mentioned only that of Adamson. The latest report is that of Giri (39), 1936, who found a salivary phosphatase and observed that it was identical with the urinary phosphatase reported by Dmochowski and Assenhajm (24). Giri examined the paraffin activated saliva of adults collected with toluol, centrifuged and dialyzed. He described the enzyme as an added glycerophosphatase, having an optimum pH range of 4.0 to 5.0.

It becomes evident from the findings for all phosphorus fractions, including the enzyme phosphatase, that definite conclusions for establishing standard norms of ranges for saliva cannot be reached. The scarcity and inadequacy of results reported in the literature is

disconcerting and bewildering and opens a wide field for scientific investigation.

SUMMARY AND CONCLUSIONS

The increasing interest of the dental profession in salivary composition has made it necessary to study in detail those elements in the body that are closely related to growth and development of bone and teeth: calcium (11, 114) and phosphorus (12). Considerable knowledge on the importance of phosphorus compounds for the maintenance of health of the body is available; however, little, if any, information is available regarding their influence on mixed human saliva. The fact that several phosphorus fractions exist complicates the understanding of the problem considerably. The interest for further investigations has been renewed by statements of several authors to the effect that the formation of calculus and the occurrence of paradentosis and caries seem to depend largely on the variability of salivary phosphorus.

Many attempts have been made to gain insight into this important problem, but the results obtained are not too encouraging. This is true not only of the percentage distribution of the various phosphorus fractions and salivary phosphatase but also of the actual phosphorus results in milligrams per cent (12). There was more a lack of understanding of the nomenclature and differentiation of the various phosphorus fractions present in saliva than discrepancies in technique. It became necessary, therefore, to investigate carefully the technical approach of each investigator as well as to determine the actual fraction or fractions examined. In many instances it was impossible to detect from the reports what actually was done; many were directly misleading. The fundamental principles of accuracy and applicability of phosphorus analysis to general biological material have been discussed in this paper and applied to the determination of salivary phosphorus fractions. These conclusions enable one to judge the merits of the many publications which have appeared in the last few years and to utilize them for comparative purposes.

Among the investigators of salivary phosphorus there were only 12 who were interested in more than 1 fraction (Table III) and not all of

this group determined the same fractions. The results obtained by actual determination or calculation vary tremendously. Close analysis of the investigators' work leads the reader to believe that the discrepancies are due to differences in the selection of material and the preliminary treatment of saliva samples.

The inadequacy of reported material and procedures makes it impossible to establish definite normal percentage ranges for the various fractions and emphasizes the necessity for further scientifically planned investigations in this field.

REFERENCES TO LITERATURE

References for this paper and the two accompanying papers have been combined and appear at the end of the last paper (p. 247).

HUMAN SALIVA

V. A Technical Procedure for Phosphorus Analysis[1,2]

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INTRODUCTION

The technical procedure of phosphorus analysis has offered great difficulties because of the slow growth of knowledge of the phosphorus fractions and their organic combinations, and the great variations in concentration and amount of phosphorus in the material examined. Most salivary methods have been of the type not requiring preliminary precipitation of phosphorus. The continuous improvement which these methods have undergone has lately resulted in simple, speedy micro methods having an accuracy claimed to be within ±2 to 3 per cent over a range of phosphorus from 0.01 to 0.04 mg.

It has been shown (12, 12A) that most investigators of salivary phosphorus used one of the following colorimetric methods: Bell-Doisy (13) (or modifications thereof); Kuttner and Cohen (74) (or modifications thereof); Tisdall (112); or Fiske and Subbarow (34). In spite of the fact that methods were used which are accepted as accurate for the analysis of blood, the results of salivary analysis vary so much that it is not possible to compare the work of different authors. It was therefore necessary to survey the literature from the standpoint of the phosphorus fraction examined because this important point was ignored in the interpretation and description of data by many in-

[1] This research has been carried on under a Fellowship grant from the Carnegie Corporation, New York City.

[2] Presented at a joint meeting of the San Francisco Section of the International Association for Dental Research and the Northern California Section of the American College of Dentists in San Francisco, March 10, 1937, and to the California State Dental Association in San Jose, April 13, 1937.

vestigators (12). Some authors reported the analysis of more than 1 fraction.

For the establishment of normal standards, which remains the *primary* problem confronting the investigator of the minerals of saliva, the first requisite is a method applicable to saliva. In order to be sure that all of the possibilities of technical error are adequately controlled, it is necessary to check the several types of methods accepted in general biochemical work on test solutions containing known amounts of phosphorus and on saliva itself.

APPLICATION OF THE FUNDAMENTALS OF PHOSPHORUS DETERMINATION TO THE ANALYSIS OF TEST SOLUTION AND SALIVA

A. Test Solution. The variation in results reported in the literature is, in part, due to the many deficiencies in the approach to salivary phosphorus analysis (12, 12A). The checking of a method on a test solution has been considered minor, yet it is very valuable in judging the accuracy of a method in the hands of the individual technician and its suitableness for the amounts of phosphorus contained in the samples available. It is surprising to note that in 44 reports only 8 stated that the method had been checked on a test solution. Mathis (82) even stated that the method he used was so well described by the original authors that it was not necessary for him to check it on test solution. In order to check the accuracy in our hands of the 4 most popular technics (12), the following were tried on test solution and saliva: Tisdall; Briggs, Bell-Doisy: Fiske and Subbarow; and the stannous chloride method according to Bodansky. The test solution was prepared to contain 20 mg. per cent inorganic phosphorus (La Motte's buffer grade KH_2PO_4); diluted with equal parts of 10 per cent CCl_3COOH; filtered; and aliquots were taken.

The fundamental principles of each of the four methods are described separately (12A). The Tisdall method (112, 89 and 62) (recommended for saliva (71) requires between 0.02 and 0.1 mg. phosphorus (0.25 cc. test solution). The Briggs, Bell-Doisy (18, 13 and 89) procedure and the Fiske and Subbarow (34 and 43) technic are quite similar and are by far the most popular of all microcolorimetric methods in biological work. They require about 0.02 to 0.1 mg. phosphorus, corresponding to 0.25 cc. of test solution. The stannous

chloride methods have been given a recent impetus by the publications of Kuttner and Cohen (74); Kuttner and Lichtenstein (75); Youngburg (118, 119 and 44) and Bodansky (16). This method requires 0.01 to 0.04 mg. phosphorus (0.1 cc. test solution).

Each method was tested on the wet ashed and unashed (direct) trichloracetic filtrate [by cautiously heating with H_2SO_4 over a microburner; the procedure with perhydrol (Merck) in place of HNO_3 is used (see footnote page 241) (118, 89, page 69)]. The amount of H_2SO_4 used in wet ashing is adjusted so that the aliquot will contain the equivalent of 1 cc. 2N H_2SO_4. The excess acid is compensated for

TABLE I

Analyses of test solution containing 20.0 mg. per cent phosphorus by four colorimetric methods

METHOD	TRICHLORACETIC ACID FILTRATE	
	Unashed	Ashed
	mg. per cent	*mg. per cent*
Stannous chloride	20.0	20.1
	20.0	19.8
Briggs, Bell-Doisy	19.9	20.0
	20.0	19.9
Fiske and Subbarow	20.1	20.2
	20.1	20.1
Tisdall	20.0	20.0
	20.3	20.0

by the use of a second molybdate solution in which the H_2SO_4 concentration has been reduced appropriately.

The results tabulated in Table I show a satisfactory recovery of the phosphorus known to be present by each of the 4 methods. The ashing procedure is shown to be satisfactory and not to cause the loss of any phosphorus, e.g., by overheating at the meniscus, etc. and proves that no phosphorus is contained in the reagents.

B. *Saliva.* In order to test the properties of these methods on the unknown solution, saliva, 7 samples were analyzed simultaneously by all 4. In Table II are found the results by the methods of Bodansky; Briggs; and Fiske and Subbarow. Close checks were obtained

with all the methods on the same sample of saliva. Especially is it interesting to note that this is true of the unashed samples, too, because of the possible presence of organic material or other substances which could interfere with color production. In Table III are given the results by the Tisdall method. These were placed separately as they did not show as good check on the duplicate determinations as did the first 3 methods. They are sufficient to indicate check results for those of Table II. The reason for the discrepancies noted may be due to the lack of experience with the method or to the complicated procedure.

Poor color development is frequently mentioned in the literature. It may be due to a variety of causes, which include the following: (a) opalescence of the trichloracetic filtrate; (b) turbidity after the addition of the molybdate solution; (c) turbidity after the addition of the reducing reagent; (d) interference with intensity or shade; (e) instability of color. The question arises, of course, as to whether the presence of a poor color can be ignored or whether some methods are exempt from this disturbance. Several opinions have been expressed in the matter; all of them refer to the incomplete precipitation of proteins by trichloracetic acid as being a responsible factor. For this reason, 6 persons were selected whose saliva was known to produce a poor color.

From Table IV it can be seen that the 2 methods (Briggs, Bell-Doisy; and Fiske and Subbarow) which were mentioned in the literature as occasionally developing a poor color showed this defect in every one of these selected cases. The stannous chloride reagents were notably free of the defect in all but 1 of the 6 cases. By all methods the wet ashed samples were entirely free of interfering substances. It was apparently possible for the poor colors to be approximated more or less successfully with the standard since the results by all 3 methods check very closely, the percentage variation being within ± 5 per cent. It is desirable that the poor color be avoided in some way in order to facilitate the comparison of unknown and standard in the colorimeter. Clark and Shell (21) noted that, in their 5 cases, the slight turbidity of the trichloracetic filtrate disappeared upon the addition of the Bell-Doisy reagents. Forbes

TABLE II

Comparative study of phosphorus methods on the same sample of mixed resting saliva

PHOSPHORUS FRACTION	1		2		3		4		5		6		7	
	Inorganic	Total acid-soluble	Inorganic	Total acid-soluble	Inorganic	Total acid-soluble	Inorganic	Total acid-soluble	Inorganic	Total acid-soluble	Inorganic	Total acid-soluble	Inorganic	Total acid-soluble
$SnCl_2$.....	12.3	12.2	13.3	12.6	11.1	11.5	12.3	12.6	8.4	8.7	9.0	9.5	8.4	8.4
	12.2	11.8	13.0	12.6	11.2	11.6	12.4	12.6	8.3	8.9	9.0	9.5	8.2	—
Briggs, Bell-Doisy	12.9	12.5	13.1	13.3	11.7	12.2	13.0	13.0	9.4	9.6	9.3	9.7	8.4	8.9
	13.0	12.8	—	13.5	11.8	12.2	12.9	12.8	9.4	9.8	9.4	9.7	8.5	9.0
Fiske, Subbarow	13.1	12.8	13.3	14.2	12.8	13.4	13.0	13.1	9.9	9.9	9.9	10.7	8.9	9.1
	13.0	12.9	13.6	13.7	12.8	13.5	13.2	12.8	9.6	9.7	10.1	10.2	8.9	9.2

TABLE III

Analysis of salivary inorganic phosphorus and total acid-soluble phosphorus by the Tisdall method

NUMBER	INORGANIC	TOTAL ACID-SOLUBLE
	mg. per cent	*mg. per cent*
1	14.4	12.3
	14.3	12.1
2	13.3	12.5
	13.2	12.3
3	12.1	12.0
	12.9	11.5
4	13.5	14.7
	13.0	14.6
5	10.8	10.3
	10.8	10.6
6	9.8	9.3
	9.7	9.9
7	8.1	9.6
	8.4	7.7

(35) found that with the adapted Fiske and Subbarow method it was sometimes necessary to centrifuge out interfering substances. Smith (110) noted that a turbidity always developed upon the addition of the molybdate reagent in the Fiske and Subbarow; and Briggs methods, in spite of variation of the percentage of trichloracetic acid or the use of other precipitants. Smith was unable to identify the precipitate producing turbidity as a protein. He cited Robinson and Soames, who found a similar precipitate in the determination of organic phosphates in rat's blood to be due to the presence of protein

TABLE IV

Comparative study of stannous chloride; Briggs, Bell-Doisy; and Fiske and Subbarow procedures on selected cases developing poor color

	8		9		10		11		12		13	
	Inorganic	Total acid-sol.	Inorganic	Total acid-sol.	Inorganic	Total acid-sol.	Inorganic	Total acid-sol.	Inorganic	Total acid-sol.	Inorganic	Total acid-sol.
$SnCl_2$.....	12.3	12.9	16.6	17.0	16.1	16.3	10.7	11.1	9.1	9.0	20.5B	20.1
	12.1	13.1	16.8	17.0	16.0	16.5	10.3	11.1	9.1	9.1	20.5A	19.9
B, B, D...	12.1D	13.5	16.3A	18.3	16.0A	17.3	10.5B	11.2	9.5E	9.0	19.5E	19.6
	12.1D	13.8	16.4A	18.3	16.5A	17.4	10.5B	11.2	9.1E	9.1	20.3E	19.9
F and S...	12.1B	13.0	15.8A	17.1	15.9A	17.4	10.5C	11.0	9.2C	9.8	19.7C	19.9
	12.0C	13.3	15.8A	17.0	15.9A	16.8	10.5C	11.1	9.1C	9.5	19.4C	19.7

A—very slight greyish blue color, B—slight greyish blue color, C—greyish blue color, D—greyish green color, E—extreme greyish green color.

cleavage products. Smith found that a preliminary isolation of the phosphorus as magnesium ammonium phosphate enabled him to remove all interfering substances. Krasnow, Karshan and Krejci (71) reported that lower results were obtained on trichloracetic filtrates when analyzed by the Fiske and Subbarow technic than when the Tisdall procedure was used. Krasnow (70) suggests that the Tisdall procedure is not affected by the slight opalescence of some trichloracetic filtrates as is the Fiske and Subbarow technic. Shear and Kramer (107) removed the interference with color development by turbidity, in the analysis of bone trichloracetic filtrates by the

Briggs, Bell-Doisy technic, by filtering the molybdate solution just before adding the sulfite and hydroquinone.

In the case of saliva none of these measures seem adequate for the control of poor color development with the Bell-Doisy or Fiske and Subbarow technics. Clark and Shell's experience was not confirmed on a larger number of cases (see Table IV). Forbes' method of centrifuging out the suspended material of the final colored solution was found to give low values, as was Shear and Kramer's technic of filtering the turbid molybdate solution. The use of alcohol as a precipitant also resulted in loss of phosphorus. When these samples developing poor color are encountered, it is possible to wet ash[3] the trichloracetic filtrate, thus determining the total acid-soluble phosphorus instead of the inorganic fraction. This unsatisfactory situation can usually be avoided by the use of the stannous chloride reagents, as described by Bodansky. The blue color developed by these reagents is so much more intense than that produced by the Briggs or Fiske and Subbarow procedures that only 4/10 the amount of saliva is required to produce a color of about double intensity.

Because of the consistent accuracy, simplicity and speed of the stannous chloride method, as described by Bodansky (16), it has been selected, with certain modifications, as the standard method for this laboratory.

Preliminary centrifugation has not been used by many investigators in the analysis of salivary phosphorus. As was the case with calcium (11 and 114), it was necessary to decide whether or not to include in the analysis of saliva the epithelial cells, debris, etc. Of the 44 authors reporting the analysis of salivary phosphorus (12) only 3 (19, 117, 9 and 10) used preliminary centrifugation. Clark and Levine (20)

[3] Transfer aliquots to a pyrex 10 cc. volumetric flask and reduce volume to few drops by boiling cautiously over micro burner. Add 0.5 cc. 10N H_2SO_4 and heat until white fumes appear in flask but not hotter in order to prevent loss of H_2SO_4 or H_3PO_4. Continue heating until mixture darkens as much as it will, allow to cool about 30 seconds; then add drop of perhydrol and heat. More perhydrol is used if the solution does not clear (89, page 69; 118). By heating cautiously from the side of the flask the use of a bead to prevent bumping is avoided. The samples may be dry-ashed in platinum by simple ignition (89, page 70). In this case, the dry ash is dissolved by the equivalent of 0.5 cc. 10N H_2SO_4 and made to volume in a 10 cc. volumetric flask. Aliquots of 4 cc. are then taken and a molybdate is used which has been corrected for H_2SO_4 content.

discarded the use of preliminary centrifugation after testing phosphorus (as well as calcium and chlorides) on both centrifuged and uncentrifuged saliva. They found a loss due to centrifugation. In a footnote they state that this is not likely to be due to the loss of organic phosphorus through the removal of epithelial cells, etc., because of ". . . the fact that all the phosphorus of the saliva is present in the inorganic form . . ." They refer here to the work of Clark and Shell (21). The latter actually found that the *inorganic phosphorus* of saliva *equaled* the *total acid-soluble fraction*. This has no bearing in the problem of preliminary centrifugation as *it does not apply to the total phosphorus* of saliva *but only* to the *total acid-soluble fraction*. Clark and Levine determined the *total phosphorus* of saliva which includes in addition to the total acid-soluble phosphorus the *organic fraction insoluble in acid*, for which they presented no data. Therefore, they have not removed the possibility that the higher phosphorus values of uncentrifuged saliva, as compared with centrifuged saliva, may be due to the inclusion of phosphorus from cells, debris, etc. Becks (10) stated that this additional step of preliminary centrifugation is necessary in order to remove any influence of debris and cells upon the result. To check the influence of centrifugation on the results, 5 saliva samples were analyzed for the total phosphorus, total acid-soluble and inorganic phosphorus content. The results are recorded in Table V. Both methods of dry and wet ashing were checked against each other and it is interesting to note the agreement between them. Decreases were found in the total and total acid-soluble phosphorus of the centrifuged samples similar to those noted in calcium analysis. The exceptions are few, and it can be concluded generally that the centrifugation process has a very definite influence on the results. A decided difference in the inorganic fraction of centrifuged and uncentrifuged samples could not be found. It is, however, conceivable that in certain samples a contamination by insoluble inorganic phosphorus can occur which later would become dissolved by the trichloracetic acid reagent and would not only give higher values for total phosphorus and total acid-soluble phosphorus, but also for inorganic phosphorus in contrast to the centrifuged sample. It must be admitted that for the determination of the organic fractions, the centrifugation process probably eliminates a

small and variable quantity of organic material other than that contained in cells.[4] However, since the centrifugation procedure is the only one which allows the removal of contaminating material, it is our belief that a sacrifice has to be made at the expense of the organic fractions.

Comparative percentages for the above 5 cases (Table V) were calculated using the total phosphorus of the centrifuged saliva as 100 per cent. The average percentages for these 5 cases are total phos-

TABLE V

*Influence of preliminary centrifugation on the determination of total phosphorus, total acid-soluble and inorganic phosphorus of mixed resting saliva**

PHOSPHORUS FRACTION	1		2		3		4		5	
	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged
Dry ashed total P	11.5	10.7	32.1	30.1	34.1	33.8	15.4	14.8	11.8	11.1
	11.5	10.7	32.6	30.1	33.5	33.7	15.5	14.8	11.9	11.1
Wet ashed total P	11.4	10.6	33.4	30.3	34.6	33.1	15.2	15.4	12.5	11.1
	11.6	10.4	33.2	30.2	34.3	33.1	14.9	15.4	12.2	11.1
Total acid-soluble P	11.4	10.6	31.2	30.3	33.5	33.2	15.5	15.4	11.9	11.1
	11.2	10.7	31.3	30.3	33.7	33.8	15.2	15.7	11.6	11.3
Inorganic P	10.6	10.3	29.6	28.9	30.7	31.5	13.7	13.8	11.2	10.5
	10.7	10.3	29.2	29.2	31.0	31.8	13.7	13.9	11.1	10.5

* The stannous chloride method of phosphorus analysis according to the modification of Bodansky was used throughout this series.

phorus: uncentrifuged—105.8 per cent, centrifuged—100.0 per cent; total acid-soluble: uncentrifuged—103.6 per cent, centrifuged—101.0 per cent; inorganic: uncentrifuged—96.3 per cent, centrifuged—94.9 per cent. The losses due to centrifugation were calculated from these values[5] and found to average 5.4 per cent in the case of total phosphorus, the highest differences being well outside of the range

[4] An investigation of the question of organic composition and percentage loss through centrifugation is in progress in this laboratory.

[5] Lack of space necessitated deletion of parts of the text and several tables which would have aided in understanding the conclusions drawn.

allowed for experimental error. The total acid-soluble phosphorus shows losses well within the experimental error, while the inorganic fraction shows losses which cannot be considered significant.

These findings show that there is a significant loss after centrifugation if only the total phosphorus is determined. This loss was supposed by Clark and Levine to be due to the formation of a calcium-phosphorus-mucin complex as a precepitate. This hypothesis, however, would not explain why the inorganic phosphorus fraction does not show a loss after centrifugation. What appears to happen is that only organic phosphorus is removed. In the light of these findings the hypothesis of Clark and Levine will not hold. Although there may be a slight loss by centrifugation in the total salivary phosphorus, nevertheless, there should be some routine means of removing the epithelial cells, debris, etc. It can, therefore, be generally stated that the centrifugation process as found to be essential for all calcium analyses, is also a requirement for the determination of all phosphorus fractions.

The most satisfactory method adaptable to phosphorus determination in saliva under the widest possible range of conditions, is that described by A. Bodansky in which the stannous chloride reagents are used. It can be applied where there is a wide difference of phosphorus concentrations in standard and unknown solutions. These applications were made possible when Bodansky introduced the corrected formula for calculation of the unknown. In addition, the reagents are, in general, more stable than in other colorimetric methods, and the procedure is brief and thus not subject to the hazards of the more complicated and tedious ones.

RECOMMENDED METHOD FOR THE DETERMINATION OF SALIVARY INORGANIC PHOSPHORUS

Complete descriptions have been given previously (114) for the procedures of: collection of saliva, preliminary centrifugation, deproteinization and the preparation of the trichloracetic filtrate.

After centrifugation of the saliva and preparation of the trichloracetic filtrate the colorimetric procedure is as follows: 1. Transfer with an accurately calibrated pipette 0.2 cc. trichloracetic filtrate to a pyrex 10 cc. volumetric flask. 2. To another pyrex

10 cc. volumetric flask add 2 cc. standard solution (equivalent of 0.02 mg. phosphorus). 3. To each of these flasks containing unknown and standard add water to 4 cc. in the flask. 4. Add 4 cc. molybdate reagent to each flask and mix. 5. Add 1 cc. dilute $SnCl_2$, make to volume with water. 6. After a minute or more compare in colorimeter. (Colorimetric technic is described by Peters and Van Slyke (89) page 36, and Hawk and Bergeim (44)). Calculation: the cor-

FIG. 1

rected tables and formulae of Bodansky are followed. The corrected formula is essential to the success of the method. The accompanying nomogram is used for direct calculation when the equivalent of 0.1 cc. saliva is used, as in this method. The figures for mg. per cent phosphorus are the same as those of Bodansky's Table I times 1,000.

Reagents: (a) 10 per cent trichloracetic acid (Merck). (b) Standard phosphate solution. Potassium acid phosphate (La Motte, buffer grade) prepared so that 2 cc. contain 0.02 mg. phosphorus. Preserved with a drop of toluene. (c) Molybdate reagent. Prepared

fresh daily by mixing: 1 volume of cold 10N H_2SO_4 (300 cc. concentrated H_2SO_4 made to 1 liter), 1 volume 7.5 per cent molybdate stock solution added quickly while mixing, and finally 2 volumes of water. (The 7.5 per cent molybdate stock solution is prepared in a 2 liter flask from a solution containing 90 g. molybdic acid (Eimer and Amend, T. P., Special, "Ammonia- and phosphate-free") in 250 cc. of 5N NaOH. This is made to 2 liters and mixed. The solution should be faintly alkaline to phenolphthalein. Let stand and decant for use.) (d) Dilute stannous chloride. Sixty per cent solution of stannous chloride (Eimer and Amend, T. P.) in concentrated hydrochloric acid (stored in ice-box). Dilute 1 to 200 for use daily.

SUMMARY

1. The available surveys of the literature on the calcium and phosphorus composition of mixed human saliva indicate the need for further clarification of the rôle of salivary electrolytes in oral conditions. 2. One obstacle in this process has been the lack of a satisfactory method for salivary inorganic phosphorus analysis. The adopted method must be not only thoroughly tested and compared with others of proven accuracy and worth in the biological field, but must satisfy the peculiar conditions of salivary analysis. 3. The popular methods of Tisdall; Bell and Doisy; stannous chloride; and Fiske and Subbarow were tested on known solutions of inorganic phosphate with satisfactory recoveries. When these methods were tried on saliva several difficulties were encountered which interfered with accurate checks. 4. Poor color development was pronounced by the methods of Bell-Doisy; and Fiske and Subbarow. 5. The stannous chloride procedure, as modified by A. Bodansky, was found to give the most satisfactory results over the widest possible range of conditions. 6. Bodansky's procedure adapted to saliva made possible the use of the smallest sample of saliva (average 0.1 cc.) for a single determination; gave the best color development when tested on selected cases developing poor color by the Bell-Doisy and Fiske-Subbarow technics; and was found suitable over a range of amounts of inorganic phosphorus from 0.0090 to 0.0560 mg. 7. Preliminary centrifugation of the saliva sample was adopted after testing and discussing the findings of Becks; and Clark and Levine. Losses due to preliminary centrifugation

were shown to involve principally the organic phosphorus fraction. This does not support the hypothesis of Clark and Levine, who state that the losses in centrifuged saliva are not due to the removal of cells, debris, etc., but to the formation of a "calcium-phosphorus-mucin complex." If this were true the losses would occur in the inorganic fraction as well. Clark and Levine's findings, based upon the analysis of only the total phosphorus content, were insufficient to permit this conclusion. 8. Trichloracetic acid was used to precipitate proteins and permit the determination of the inorganic phosphorus fraction. 9. A nomogram was constructed to lessen the labor of calculation. On the chart the result in mg. per cent phosphorus can be found directly from the colorimetric reading. 10. A complete description of the recommended method according to the modified Bodansky procedure is given for the determination of salivary inorganic phosphorus. Modifications for the determination of total phosphorus and the total acid-soluble fraction are also noted.

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HYDROGEN ION CONCENTRATION OF THE DENTAL PLAQUE

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Dental caries is a process of dissolution of the enamel and dentin. It begins on those areas of the tooth surface which will retain adherent material of a bacterial nature, the so-called "plaque" that was demonstrated in beginning smooth-surface caries by Williams (1). Since the enamel, which is composed of over 95 per cent inorganic substance, must be penetrated before the dentin is reached in most cases, it follows that the carious plaque must be capable of dissolving the inorganic content of the enamel.

Studies on the solubility of enamel (2, 3) have demonstrated that whereas slight dissolution of enamel is possible in buffer solutions with a hydrogen-ion concentration as low as pH 8.0; nevertheless dissolution occurs only at much greater hydrogen-ion concentrations when the buffer solutions contain small amounts of calcium phosphate. The exact physico-chemical conditions under which enamel dissolves in the mouth have never been determined but there is evidence to indicate that the pH must be about 5.0 or below. Saliva has been shown to contain sufficient calcium phosphate to prevent it from appreciably dissolving enamel at the pH at which saliva exists in the mouth. It would seem that the caries-producing plaque must have a pH considerably below the pH of saliva. The measurement of the pH of the plaque is of the greatest importance for evaluating the factors which cause or prevent dental caries.

Etherington and Trimble (4) have reported measurements of the pH of plaques made by disintegrating them in 5 cc. of redistilled water and using a colorimetric technique. The author was unable to measure the pH of a plaque by disintegrating it in 5 cc. of redistilled water and using either a colorimetric technique or the glass electrode. The chief difficulty encountered in measuring the pH of the plaque

was its size. The volume of the plaque was seldom greater than 1 cm. when removed from the surface of the tooth. As this was not sufficient material for the usual methods of measuring pH, a special microscopic technique was devised. The colorimetric method was adapted for use in a space about $\frac{1}{4}$ mm. in depth. A chamber of this depth, similar to the chamber in a hemocytometer, was made by separating a microscopic slide from a cover-glass with small sections of a second cover-glass. The plaque was placed in this chamber, a few times its own volume of indicator solution added and then buffer indicator solutions of known pH were placed in the chamber near the plaque for color comparison. The concentration of the indicator solutions was adjusted to give a good color intensity at this shallow depth and a microscope used to view the slide.

MATERIALS AND METHOD

The indicator solutions used were: .1 per cent aqueous solutions of Brom-cresol Green, Brom-cresol Purple, and Brom-thymol Blue which had been brought to their mid-points by the addition of sodium hydroxide. The buffer-indicator standard solutions were made by mixing equal volumes of .2 per cent indicator solutions with buffer solutions of Clark and Lubs of double concentration. These solutions were periodically checked by using the glass electrode, and they covered a range of pH 4.0 to 7.6 in steps of .2 pH. Special slides were prepared by cutting 4 mm. wide sections of $\frac{1}{4}$ mm. thick cover glass with a diamoned point, and cementing 4 of these sections on a microscope slide with Canada balsam so as to create 3 equal spaces between them. When the Canada balsam had hardened the slides were thoroughly cleaned and rinsed in distilled water. The cover glasses which were used over this special slide were slightly smaller than the slide covering all 3 of the spaces. A binocular dissecting scope with 20× magnification used to examine the plaque and indicator on the special slides.

Pipettes for the placing of the indicator solutions on the slide were made by drawing 4 mm. glass tubing to a fine capillary point about .1 to .2 mm. in diameter. A small piece of rubber tubing sealed with rubber cement at one end was used as a bulb on the pipette so that very small quantities of solutions might be accurately expelled into the slide compartment.

The mouth was rinsed with about 200 cc. of warm distilled water to remove as much saliva as possible. The areas to be studied were isolated with cotton rolls and excess water absorbed on cotton pledgets. As much of the plaque as possible was removed with a stainless-steel scaler and immediately placed on a special slide. If the plaque was sufficiently large it was divided into 2 or 3 parts. A second and third plaque were likewise placed in the other divisions of the slide. A small amount of indicator solution (about 1 cm.) was placed over each portion of plaque with a capillary pipette and a cover glass placed over the slide. Brom-cresol Purple was the indicator used on the first portion of the plaque. If it turned a yellow color then Brom-cresol Green was used on the second portion of the plaque; if it turned purple then Brom-thymol Blue was used on the second portion. In this way the pH of the plaque was certain to fall within the range of one of the indicators.

The slide was placed on the microscope and the color of the indicator around the plaque observed. Small quantities of the buffer-indicator standard solutions were then placed near to, but separate from, the plaque with a capillary pipette, and the colors compared. The pH of the standard that matched the color of the indicator around the plaque was taken as the pH of the plaque. All of these operations were done in the least possible time in order to avoid atmospheric influences. That this method is capable of measuring the pH of one-third cm. of plaque to an accuracy of .2 pH was shown by similar tests on buffers of known pH.

DISCUSSION

The findings have been listed in Tables I, II, III and IV. All plaques listed in Table I were removed from the buccal, labial or proxi-

TABLE I

Number of plaques at pH indicated

pH \ CASE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22 A	22 B	TOTAL
4.6																					1			1
4.8																					5	1		6
5.0			3									1									1	6		11
5.2	8		9				3													1		2	2	25
5.4	1		8				1								1				1		1	2	1	16
5.6	2		3			1	4												1		1	2	1	15
5.8	2		11				2	3		2									1		3	4	2	30
6.0		1	4	3		2	1		2	1			2		1				1			2	10	30
6.2	4		8		1	2		2	1	3						4		3	1				3	32
6.4	1	4			3				1			1				1			1		3			15
6.6		3	2		1				1				4										1	12
6.8		1			1			2	2		2	2		1			1	2				1	2	17
7.0																	1							1
Average.....	5.6	6.5	5.7	6.0	6.4	6.0	5.6	6.2	6.4	6.0	6.8	6.2	6.4	6.8	5.7	6.2	6.9	6.5	5.9	5.2	5.5	5.5	6.0	5.9
Total.....	18	9	48	3	6	5	11	7	7	6	2	4	6	1	2	5	2	5	6	1	15	20	22	211

mal surfaces of teeth. These surfaces were clinically intact and readily accessible. The carious debris (Table II) was removed from the cavity surface by scraping the surface with a scaler. All plaques and all carious debris were removed from the mouth at one sitting except in cases 1, 3, 7 and 22, from which plaques were taken on several different occasions. No attempt has been made to indicate the areas of the mouth from which plaques were taken except in case 22.

Cases 1 to 7 were children staying in the orthopedic hospital whose mouths contained one or more carious teeth and whose ages ranged from 11 to 16 years. It is possible that the change in diet produced

by living in the hospital was a factor which modified their susceptibility to caries, and since the children's stay in the hospital was seldom equal to 6 months it is probable the cavities reflected previous mouth

TABLE II

Number of open cavities at pH indicated

pH \ CASE	1	3	4	5	6	8	9	12	13	14	15	16	18	19	20	23	TOTAL
4.2								1									1
4.4											2			1		1	4
4.6				1		1				1	1				1		5
4.8																	
5.0	1	2											1				4
5.2	5	5															10
5.4	2	1					1										4
5.6	1	1	1		2							1					6
5.8		1							1								2
6.0		1															1
6.2		1															1
Average pH.....	5.2	5.4	5.6	4.6	5.6	4.6	5.4	4.2	5.8	4.6	4.5	5.6	5.0	4.4	4.6	4.4	5.2
Total.....	9	12	1	1	2	1	1	1	1	1	3	1	1	1	1	1	38

TABLE III

Comparison of total number of plaques with total number of cavities at pH indicated

pH	4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8	7.0	TOTAL	pH AVERAGE
Total plaques....	0	0	1	6	11	25	16	15	30	30	32	15	12	17	1	211	5.9
Total cavities....	1	4	5	0	4	10	4	6	2	1	1	0	0	0	0	38	5.2

TABLE IV

Comparison of average pH of plaques and carious débris in 15 cases

CASE	1	3	4	5	6	8	9	12	13	14	15	16	18	19	20	AVERAGE
Average pH plaques...	5.6	5.7	6.0	6.4	6.0	6.2	6.4	6.2	6.4	6.8	5.7	6.2	6.5	5.9	5.2	5.9
Average pH caries....	5.2	5.4	5.6	4.6	5.6	4.6	5.4	4.2	5.8	4.6	4.5	5.6	5.0	4.4	4.6	5.2

conditions. It is significant to notice that although the average pH of debris from cavities was .4 units below the average of plaques some plaques reached a pH as low the carious material, with the

exception of a child who had just come to the hospital (case 5). His plaques were at a relatively high pH but debris from a proximal cavity was the lowest pH of the entire group.

Cases 8 to 20 were children living in a receiving home. Their ages ranged from 10 to 17 years and most of them had been living at the home for several years. The difference in the average pH of the plaques and the carious debris was greater than in cases 1 to 7, namely 1 pH unit. If we assume that caries occurs at a pH of 5.0 or below, an assumption based on the enamel solubility studies previously mentioned, then about one half of the cavities were in active decay at the time of testing whereas only 1 plaque could be considered as initiating caries. Clinically none of the children in this group could be considered very susceptible to caries.

Case 21 offers an illustration of the possible variations in pH of plaques which were taken from different areas of the same mouth at the same time. The variation in the pH of the plaques ranged from 4.6 to 6.4. The mouth was susceptible to caries as was shown by the development of 3 small cavities during the previous year.

Case 22 was the author's mouth. At the time this study was begun there had been no caries for the previous 8 years, but 16 months later a small shallow cavity had developed on the buccal surface of the upper left third molar. Since this particular surface is most difficult to keep clean, and since no other caries has occurred in the mouth during this time, it appears significant that the plaques from this area (listed in Table I, Case 22, Column A) which later developed a cavity averaged .5 pH below plaques from other areas of the mouth (listed in Table I, Case 22, Column B) and were the only ones to reach a pH of 5.0 or below. The fact that plaques from this area varied from pH 4.8 to 6.8 suggests that the carious process was active only part of the time.

Case 23 was a measurement of carious debris from under a large gold crown. The enamel and dentin were softened extensively but retained their original form to a marked degree. The pulp was not exposed. Since mouth fluids had access to the carious area only through a small hole in the gold crown, the pH of 4.4 can be accepted as that pH at which the caries occurred.

It is obvious that within the limits of error of the method, the exact

pH of the most acid parts of the plaques or cavities was never greater than the pH as measured. It is also obvious that the measurements cover a range which might be expected to exist in the mouth, namely from a pH which might readily be expected to dissolve the enamel to a pH approaching that of the saliva and mouth tissues. Since a definite attempt was made to take plaques only from intact surfaces of teeth, the low proportion of plaques with a pH of 5.0 or below would fit in fairly well with the expected chances of finding an actively carious area. The relatively low pH of actively carious lesions fulfils the requirements for decalcification as indicated by solubility studies. The finding that at any given time the plaques of the mouth can differ by as much as 2 pH units demonstrates the effect of the localizing power of each plaque and illustrates that saliva is unable to neutralize the acids that cause decay of the teeth.

SUMMARY

211 plaques were tested for their hydrogen-ion concentrations and were found to vary from pH 4.6 to pH 7.0. The average pH was 5.9 but the distribution was fairly even from pH 5.0 to pH 6.8.

Debris from 38 tooth cavities was tested for hydrogen-ion concentration and was found to vary from pH 4.2 to pH 6.2. The average was pH 5.2. This was .7 pH below that of the plaques.

CONCLUSIONS

1. A method has been devised by which the hydrogen-ion concentration of the dental plaque can be measured.

2. The ability of the plaque to reach a hydrogen-ion concentration as low as pH 4.6 has been demonstrated. Enamel solubility studies have indicated that enamel will actively dissolve at this pH under mouth conditions.

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